Aus dem Institut für Mikrobiologie und Hygiene
Medizinische Fakultät Charité
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The Kuvin Center for the Study of Infectious and Tropical Diseases
The Hebrew University, Hadassah Medical School
Jerusalem, Israel

DISSERTATION

PCR Diagnosis of Leishmaniasis in Israel and the West Bank

Development of a field applicable procedure useful for epidemiological studies

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von

Gerlind Anders

Dekan: Prof. Dr. Joachim W. Dudenhausen

Gutachter: 1. Prof. Dr. med. W. Presber
2. Prof. Dr. med. W. Solbach
3. Prof. Dr. R. Lucius

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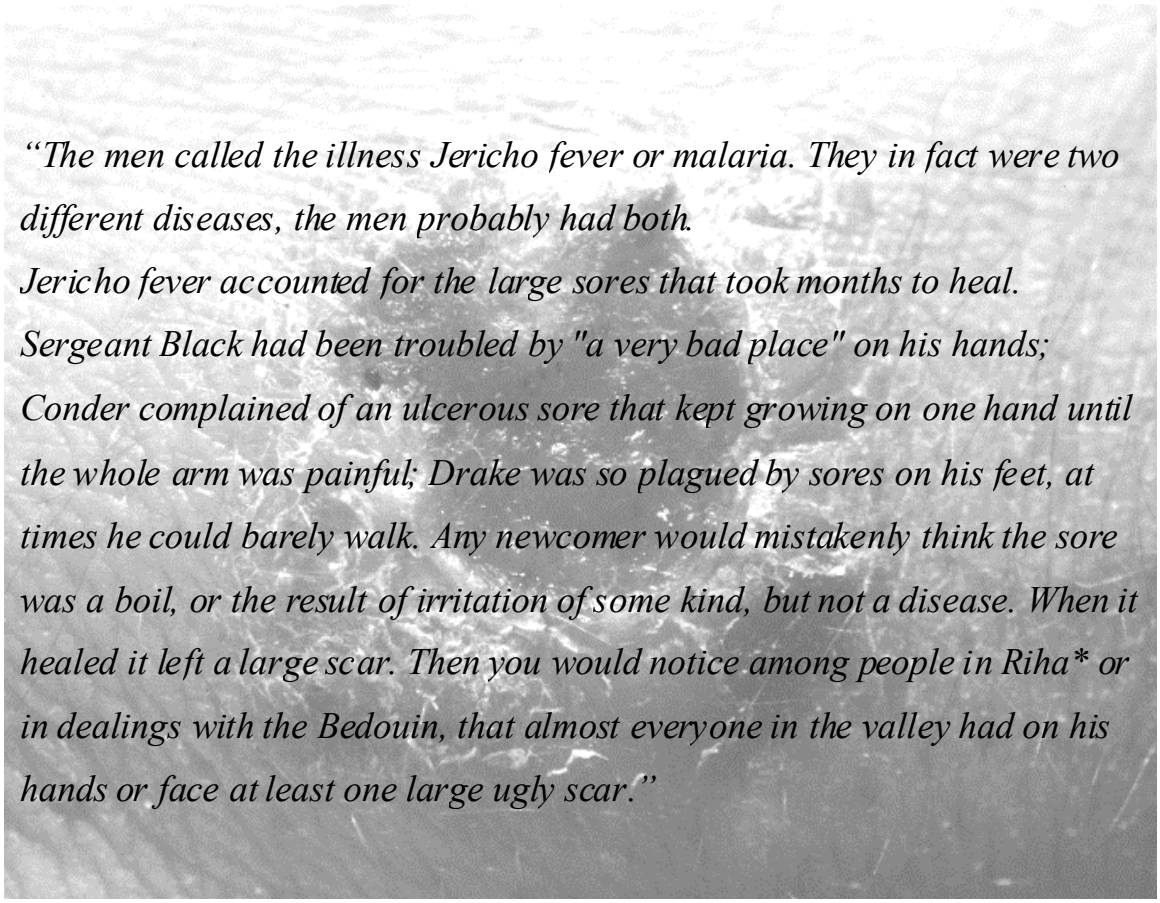
The laboratory work has been carried out at the Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University, Hadassah Medical School, Jerusalem, Israel

Travel report from Palestine, 1873



"The men called the illness Jericho fever or malaria. They in fact were two different diseases, the men probably had both.
Jericho fever accounted for the large sores that took months to heal. Sergeant Black had been troubled by "a very bad place" on his hands; Conder complained of an ulcerous sore that kept growing on one hand until the whole arm was painful; Drake was so plagued by sores on his feet, at times he could barely walk. Any newcomer would mistakenly think the sore was a boil, or the result of irritation of some kind, but not a disease. When it healed it left a large scar. Then you would notice among people in Riha or in dealings with the Bedouin, that almost everyone in the valley had on his hands or face at least one large ugly scar."*

in:

Jericho
Dreams, Ruins, Phantoms
by Robert Ruby

*Riha (Arabic) = Jericho

Table of Contents:

Tables

Figures

Photography 78-84

List of abbreviations:

BO	Bolivia
bp	Base paires
BSA	Bovine serum albumin
C.	*Crithidia*
CL	Cutaneous leishmaniasis
CVL	Canine visceral leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
ddH2O	Double distilled water
DFG	Deutsche Forschungsgemeinschaft (German Science foundation)
DMSO	Dimethyl-sulfoxid
EDTA	Ethylene diamine tetra acid
EF	Excreted factor (serological method)
ELISA	Enzyme linked immuno sorbent assay
EMBL database	European molecular biology laboratory database
ETOH	Ethanol
fg	Femtogram
G.	*Gerbillus,* gerbil
GTC	Guanidinium thiocyanate
HCl	Hydrochloric acid
IFA	Immune fluorescent assay
IFN-γ	Interferon-γ
ITS	Intergenic transcribed spacer
kDNA	kinetoplast-DNA
LRC	*Leishmania* Reference Center
L.	*Leishmania*
M.	*Meriones,* jird
MCL	Mucocutaneous leishmaniasis
NNN-medium	Novy-MacNeal-Nicolle medium
P.	*Psammomys,* fat sand rat
Ph.	*Phlebotomus,* sandfly
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PPIP-PCR	permissevely primed intergenic polymorphic PCR
RFLP	Restriction fragment length polymorphism
rpm	Rotations per minute
SDS	Sodium dodecyl sulfat
s.l.	*sensu lato*
sp.	Species
SS-media	Semi-solid media
ssp.	Subspecies
ssU RNA	small subunit RNA
T.	*Trypanosoma*
TAE buffer	Trisacetate-EDTA
Taq-polymerase	Thermus aquaticus polymerase
TH-cells	T-helper cells
U	Unit
VL	Visceral leishmaniasis
WHO	World Health Organization

1. Introduction

The following passages will give a general overview on leishmaniasis worldwide and specifically in Israel and the West Bank. The disease will be presented in its various clinical forms, in relation to the different causative *Leishmania* species. This will provide a background for understanding why sensitive and species-specific diagnosis is important. Consecutively, the facts about the biology and ecology of the parasite will be presented, which will convey a more profound understanding of the complexity of the disease. This is intended to illustrate that improved diagnostic tools are required in order to understand the epidemiology of the disease, which is a prerequisite for effective control measures. In the review of diagnostic methods the most important methods from past to present will be presented, including comparative aspects. By understanding the benefits and limitations of each of the methods presented, the selection of the specific diagnostic tools for this study will be comprehensible.

1.1. General facts about leishmaniasis:

1.1.1. Epidemiology:

Leishmaniasis is a vector borne disease caused by various members of the genus *Leishmania*, a protozoic parasite. The clinical presentation ranges from simple cutaneous lesions to life threatening visceral forms. The disease is endemic in many tropical and subtropical countries around the world (more than 80 countries according to the WHO). Leishmaniasis is not only widely distributed in warm countries, but it is also prevalent in very different topographic areas. It is endemic in rain forests (Bolivia, Brazil), deserts (Middle East, North Africa), in the countries bordering the Mediterranean Sea and also in elevations of several thousand meters (Peruvian Andes, Ethiopian highlands).

According to WHO estimates 350 million people are at risk world wide and 12 million people are affected. The annual incidence is estimated at 1-1.5 million new cases of cutaneous leishmaniasis (CL) and 0.5 million cases of visceral leishmaniasis (VL). The disease is greatly underreported, with only 600,000 officially declared cases annually. In most of the endemic countries leishmaniasis is not a reportable disease (http://www.who.int/emc/diseases/leish/leisdis1.html). The incidence worldwide is on the rise. New endemic foci have emerged over the past decades, epidemics are not controlled and endemic areas are spreading due to development and population shifts (Desjeux, 1999). In western countries the incidence is increasing due to HIV-*Leishmania* coinfection and tourism. In recent years coinfection with HIV became a serious threat in south-western Europe with 1.5-9.5% of AIDS patients being affected.

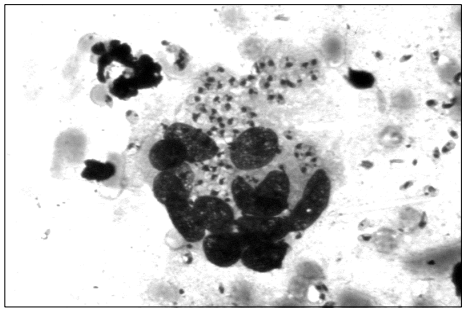
For the following passages on the parasite, the clinical picture, the transmission and control of leishmaniasis Manson's Tropical Diseases (1987; 1996) served as the main source of information, if not cited otherwise.

1.1.2. The parasite:

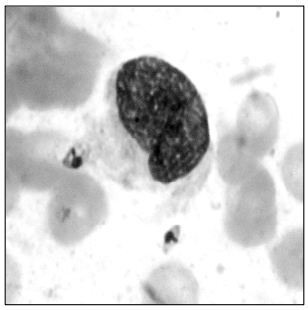
The *Leishmania* parasite is a protozoa belonging to the order *Kinetoplastida* and the family of *Trypanosomatidae*. The genus *Leishmania* includes more than 20 species. The parasite exists in two morphological forms: the nonflagellated amastigote (3-5 µm in diameter) living intracellular in macrophages of the mammalian host, and the flagellated promastigote (15-30 µm in length, plus the flagella), living extracellular in the intestinal tract of the sandfly-vector. In the macrophages the amastigotes are able to survive and multiply within the acidic phagolysosomes of the host cells (reviewed by Alexander *et al*., 1999). After multiplication in the host cell the amastigotes are released. Subsequently other macrophages are infected and the infection spreads (reviewed by Peters and Killick-Kendrick, 1987; Rittig and Bogdan, 2000).

The parasite contains two prominent organelles, the nucleus and the kinetoplast. The kinetoplast is found in all protozoa of the order kinetoplastidae (eg. *Leishmania, Trypanosoma, Crithidia*). It is a rod-shaped mitochondrial structure consisting of a DNA network of about 10,000 minicircles and about 50 maxicircles, the kinetoplast-DNA (kDNA). The function of the kinetoplast has not been clear until recently. It was found that maxicircles encode for mitochondrial ribosomal RNAs. The minicircles play a role in the editing process of these mRNAs (reviewed by Shlomai, 1994). Figure 1a and 1b show amastigotes in Giemsa stained smears, Figure 2 shows promastigotes in the sandfly gut (electron micrograph).

Figure 1: Amastigotes in Giemsa stained smears

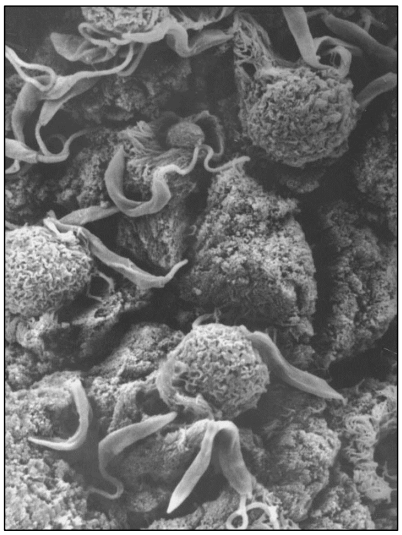


a) amastigotes in group of macrophages



b) extracellular amastigotes with prominent kinetoplast

Figure 2: Promastigotes in sandfly gut



Electron micrograph by Alon Warburg

1.1.3 Life cycle:

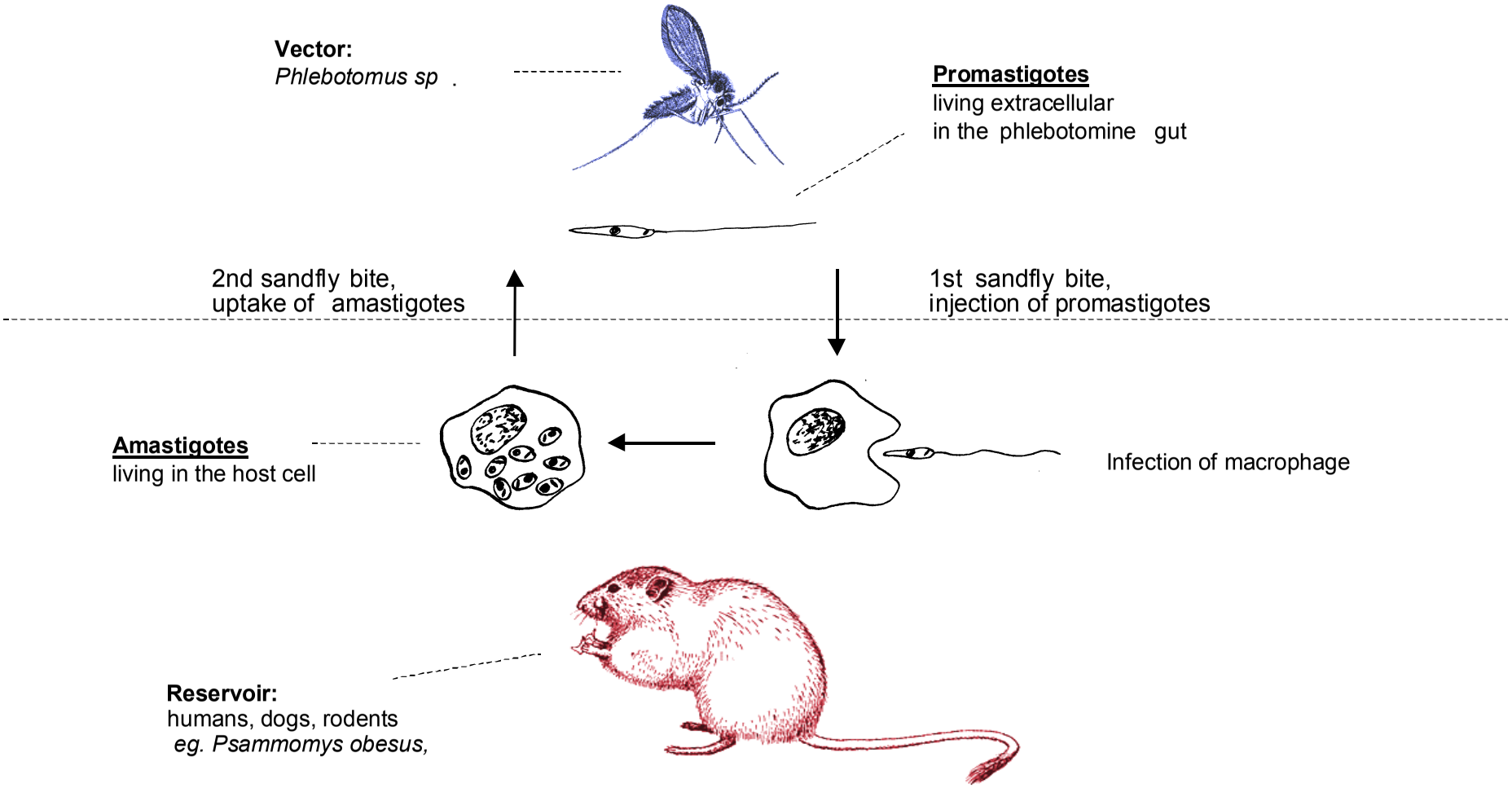
The transmission cycle is maintained between the vector and the reservoir. Depending on the species of *Leishmania* the transmission is either zoonotic or anthroponotic, involving either animals or humans as reservoir. The parasite is transmitted by the bite of female sandflies of the genus *Phlebotomus* and *Lutzomyia.* During the blood meal *Leishmania* infected macrophages are ingested by the vector. In the gut of the sandfly the intracellular amastigotes develop into flagellated promastigotes at an ambient temperature of 24-28°C. During another blood meal the mature promastigotes are inoculated into a mammalian host. Macrophages ingest the parasites, which then transform into the amastigote form. The life cycle is shown in Figure 3.

1.1.4. Clinical forms of leishmaniasis:

The three distinct forms, cutaneous (CL), visceral (VL) and mucocutaneous leishmaniasis (MCL) are classically caused by a spectrum of different *Leishmania* species each. Even though there is a clear correlation between the causative species and the clinical presentation many variations are seen. Depending on the specific characteristics of species/strains and also on the immunocompetence of the host the clinical manifestation may vary to a great extent. Species causing typically CL may visceralize and visceral species may show dermatotropism. In many endemic areas of the world a few *Leishmania* species are prevalent simultaneously so that a species specific diagnosis can not rely on clinical findings alone. Species specific diagnosis is necessary for adequate treatment.

Figure 3:

Life cycle of *Leishmania* parasites (*L.major*)



1.1.5. Immunology:

Apart from the characteristics of species and strains, the host immunology influences the severity of the disease to a great extent. Genetically determined immunological patterns of reaction, the present health condition and the nutritional status play a decisive role in the course of the disease. The events responsible for resistance or susceptibility occur early in infection. Resistance depends on a Th1-type response resulting in the production of γ-interferon (IFN-γ) and nitric oxide (NO). A Th2-type response is related to susceptibility (Blackwell, 1996; reviewed by Alexander *et al*., 1999; reviewed by Solbach and Laskay *et al*., 2000). The severity of the disease depends as well on factors related to the causative agent itself and the sandfly vector. The antigenicity is variable among strains of the same species, leading to atypical clinical presentations (eg. visceralizing *L.tropica* strains and dermatotrophic *L.d.infantum* and *L.d.chagasi* strains). Apart from this also the composition of the sand fly saliva influences the severity of the infection (reviewed by Kamhawi, 2000).

1.1.5.1. Cutaneous Leishmaniasis:

Old World:

In the Old World, cutaneous leishmaniasis (CL) is also known as "Oriental Sore", "Baghdad boil", "the little sister" (Aljebori and Evans, 1980), "Delhi boil" or "Rose of Jericho". CL is usually caused by *L.major, L.tropica* and *L.aethiopica* (see Table 1). CL presents usually as a self-limited, ulcerative skin lesion. The uncovered parts of the body, especially the face, are affected. After an incubation time of several weeks to several months a papule develops at the site of the sandfly bite, which later evolves into an ulcer. The size of the ulcer can reach a diameter of several centimeters. It lasts normally for a few (3-4) months but it can also persist for more than one year before it resolves spontaneously. The lesion is usually painless unless a secondary bacterial infection is present. A slightly depressed and often hypopigmented scar remains. If left to self-cure, life-long immunity is usually acquired against the same species. *L.tropica* infections tend to be more severe than those of *L.major* and have a potential to recidivate and also to visceralize (Magill *et al*., 1993; 1994; Sacks *et al*., 1995). As an unusual causative agent *L.d.infantum* has been reported to cause CL, eg. in Tunisia (Gramiccia *et al*., 1991), in Lebanon (Nuwayri-Salti *et al*., 1994) and in Iran (Hatam *et al.* 1997).

New World CL:

In the New World, CL is caused by species of the *L.mexicana* and the *L.braziliensis* complexes (see Table 1). *L.mexicana* infections are normally uncomplicated and self-limited. If the ear is infected, a so-called Chiclero's ulcer may develop which is characterized by destruction of the

cartilage and disfigurement of the ear. The infections caused by species of the *L.braziliensis* complex primarily present as CL. Especially the *L.braziliensis* infections are related to a high risk for later progression to mucocutaneous disease.

An atypical form of CL has been reported from Honduras (Ponce *et. al.*, 1991; Noyes *et al*., 1997) and from Nicaragua (Belli *et al*., 1999) caused by *L.d.chagasi,* which normally causes VL. In this atypical presentation the symptoms are confined to the skin. The lesions are nodular and long-lasting, resembling leprosy.

Chronic forms of CL:

Leishmaniasis recidivans or lupoid leishmaniasis is one chronic form of CL caused by *L.tropica* (Salman *et al*. 1999). Self-cure is not completed and new eruptions evolve at the border of the scarring lesion over many years. The appearance resembles lupus vulgaris. Parasites are scarce and the chronic skin lesion is mostly due to a hyperreactive immune response.

Diffuse CL (DCL) is another chronic form of CL. The infection spreads over large areas of the body. It represents a condition of anergy with a failure of cell-mediated immune response (negative Leishmanin test) and an abundance of amastigotes. In the Old World it is associated with *L.aethiopica* infections and is mostly seen in Ethiopia. It resembles lepromatous leprosy, which often led to misdiagnosis in the past. As a result, DCL patients were often kept in leprosaria by mistake. In the New World DCL is caused mainly by *L.amazonensis* (30% of the infections result in DCL). DCL occurs also in immunodeficient patients with no species-specific relation; coinfection with HIV is the most common cause (Ramos-Santos *et al*., 2000; Gillis *et al*., 1995).

1.1.5.2. Visceral leishmaniasis (kala azar):

Visceral leishmaniasis (VL) or kala azar is usually caused by species belonging to the *L.donovani* complex (*L.donovani, L.d.infantum, L.d.chagasi*, see Table1). *L.donovani* is the causative agent in the highly endemic areas of India and Sudan. In Middle Eastern and Mediterranean countries *L.d.infantum* prevails. In this region of the world mostly infants and immunocompromised adults suffer from the disease. In the New World *L.d.chagasi* is the only known species causing VL.

VL is a severe systemic disease presenting with fever, splenomegaly and cachexia. Typical laboratory findings are pancytopenia, albumin deficiency and hypergammaglobulinaemia. If not treated patients usually die after approximately two years. Often super-infections, typically tuberculosis, pneumonia or bacterial dysentery, are the lethal causes. Most infections are subclinical, only 3% of infections lead to the full picture of the kala-azar syndrome.

Rarely VL can be caused also by *L.tropica*. In India *L.tropica* has been isolated from 4 patients suffering from classical kala azar (Sacks *et al*., 1995). Visceral infections caused by *L.tropica* have been reported from veterans of Operation Desert Storm. The onset of symptoms was more acute, presenting with fever, abdominal pain, fatigue and cough (Magill *et. al.,* 1993). No cutaneous manifestations were seen and the disease was described as being viscerotropic but not identical with kala azar (Kreutzer *et. al.,* 1993).

Cutaneous manifestations related to VL:

The site of inoculation is often symptomatic and is known as leishmanioma. A small lesion appears about 3-4 months before the onset of kala azar, which should not be mistaken for CL (Adler, 1964).

Post kala azar dermal leishmaniasis (PKDL) is a form of CL occurring in some patients after treatment and cure of VL, usually after a latent phase of several years. PKDL presents as a rash, which may last for many years and may spread over large parts of the body. It can appear either as a macular, maculo-papular or nodular rash (Zijlstra and El-Hassan 2001). Often it begins with depigmented patches, which later turn to be nodular. PKDL is a serious condition which often is disfiguring the patient (social problem); it has to be differentiated from lepromatous leprosy and other skin diseases. It is mostly seen in the north-east of India (Bengal, Bihar) where 20% of the VL patients are affected. According to Manson's Tropical Diseases, (1996) East African PKDL occurs in only 2-5% of the VL cases and it does not exist in any other endemic area of VL. Recently it was reported that PKDL affects a much higher percentage of VL patients in Sudan. Zijlstra and El-Hassan (2001) found that 55% of the VL patients in Sudan developed PKDL. In the African form of PKDL the rash appears much earlier than in the patients from India, either during active kala azar or within 6 months after cure.

1.1.5.3. Mucocutaneous Leishmaniasis (Espundia):

Mucocutaneous leishmaniasis (MCL) or Espundia is almost exclusively seen in Central and South America. It is a chronic and very serious condition, developing years after self-cure of cutaneous lesions. The infection causes a progressive destruction of the mucosa, the cartilage and bones of nose and pharynx, leading to a severe mutilation of the face. MCL is mainly caused by *Leishmania* species belonging to the *L.braziliensis* complex (see Table 1), predominantly by *L.braziliensis.* The risk of developing MCL after cure of CL is estimated to be up to 40%. MCL can be lethal, often by aspiration pneumonia. Infections caused by *L.panamanensis* and *L.guyanensis* rarely result in MCL, whereas *L.peruviana* is not associated with MCL. *L.guyanensis* infections are also known as 'pian bois', typically presenting with multiple lesions.

L.peruviana infections are known as 'uta' and are usually self-healing within a few months. Infections caused by the *L.braziliensis* complex are often associated with lymphadenopathy, especially infections with *L.braziliensis* (Barral *et al*., 1992). In Sudan a less invasive form of MCL is seen in patients infected with *L.donovani*. In contrast to the American form, mucosal involvement usually precedes the clinical signs of kala azar (El-Hassan and Zijlstra, 2001).

1.1.5.4. Leishmaniasis and HIV-coinfection:

Most cases of coinfection with HIV and *Leishmania* have been reported in south-western Europe, with *L.d.infantum* being the predominant causative species. In conditions of immune suppression the clinical picture of leishmaniasis is often atypical. The typical affiliation between species and clinical presentation does not exist in many cases. Not only can cutaneous *Leishmania* species lead to systemic visceral disease, but also parasites infiltrate tissues and organs which are normally not affected. VL in HIV-positive patients has also been reported to be caused also by species which normally do not visceralize, eg. *L.braziliensis* (Hernandez *et al*., 1993), *L.major* (Gillis *et al*., 1995), *L.mexicana* (Ramos-Santos *et al*., 2000). Likewise, visceral disease (*L.d.infantum*) can be accompanied with unusual cutaneous lesions (Postigo *et al*., 1997). Alvar *et al*., (1997) have reported that *L.d.infantum*- HIV coinfections can present with all forms of leishmaniasis including MCL, PKDL and disseminated CL. In disseminated forms of leishmaniasis parasites have been repeatedly found in the digestive tract, namely in the colon (Sebastian *et al*., 1997), in the duodenum (Hamour *et al*., 1998) and in an anal ulcer (Perez-Molina *et al*., 1997). A retrospecive study in 91 coinfected patients suggests the presence of *Leishmania* amastigotes in atypical locations to be related to the immunological status (Rosenthal *et al*., 2000). The following Table 1 gives an overview on the different *Leishmania* species, the most common clinical manifestations, the geographical distribution as well as the specific host and vector species.

1.1.6. Treatment:

Simple CL due to *L.major* is mostly left to self-cure. It is even preferable not to treat, so that long-term immunity can be acquired. This especially applies to patients living in endemic areas. Treatment is necessary when cosmetically or functionally important sites are involved. *L.tropica* infections generally require treatment. For local treatment, intralesional Pentostam (sodium-stibogluconate), ketoconazol, cryotherapy with liquid nitrogen or heat can be applied. For systemic treatment, pentavalent antimonal compounds (Sb) eg. Pentostam and Glucantime are used (Herwaldt, 1999 a; Norton *et. al*., 1992).

Table 1 : Species of *Leishmania*, clinical manifestation, geographical distribution , reservoir and vector:

Complex	Species	Clinical manifestation	Geographical distribution	Reservoir	Vector
Old World:					
	L.major	CL	Western and Central Asia Middle East, Africa, India	rodents: Fat sand rat (*Psammomys.obesus*), gerbils (*Meriones, Rhombomys*)	*Phlebotomus paptasi*
	L.tropica	CL, L.recidivans	Western and Central Asia Middle East, North Africa, Sub-Saharan Savanna, India	humans, dog infections reported, hyraxes suspected	*P. sergenti*
L.donovani	*L.donovani*	VL, PKDL	India, Sudan, Kenya	humans	*P.argentipes*
L.donovani	*L.d.infantum*	VL, (CL), CVL	Mediterranean basin Middle East, China	dogs, wild canids (foxes, jackals)	*P.perniciosus, P.ariasi*
	L.aethiopica	CL, DCL	Highlands of Ethiopia and Kenya, Sudan	hyraxes	*P.longipes, P.pedifer*
New World:					
L.braziliensis	*L.braziliensis*	CL, MCL	Central and South America	forest rodents	*Lutzomyia ssp.*
L.braziliensis	*L.panamanensis*	CL	Central America, Columbia	sloths	*Lu.*sp.
L.braziliensis	*L.guyanensis*	CL	Guyana, Brazil	sloths	*Lu.*sp.
L.braziliensis	*L.peruviana*	CL	Peru, Argentine	dogs, humans?	*Lu* sp.
L.mexicana	*L.mexicana*	CL, Chiclero's ulcer	Central and South America, Texas	forest rodents	*Lu.*sp.
L.mexicana	*L.amazonensis*	CL, DCL	Brazil, Venezuela, mostly north of the Amazon	forest rodents, Opossums	*Lu.*sp.
L.donovani	*L.d.chagasi*	VL, atypical CL, CVL	Central and South America CL in Honduras, Nicaragua	dogs, wild canids	*Lutzomyia longipalpis*
Others: (Old World)					
	L.gerbilli	human infection unknown	Eastern Russia, Mongolia	Great Gerbil *(Rhombomys opimus)*	
	L.turanica	human infection unknown	Central Asia	*R. opimus*	
	L.arabica	human infection unknown	Saudi Arabia	*Psammomys obesus*	
	L.killicki		Tunisia		

This table is an extract from 3 different sources: Manson's Tropical Diseases, 19th edition (1987); *in* New Generation Vaccines: Vaccines against leishmaniasis (Eisenbeger and Jaffe, 1997); the leishmaniases as emerging and reemerging zoonoses (Ashford, 2000). CL -cutaneous leishmaniasis, VL -visceral leishmaniasis, PKDL-post-kala-azar dermal leishmaniasis, CVL -canine visceral leishmaniasis, DCL -diffuse cutaneous leishmaniasis MCL -mucocutaneous leishmaniasis, *L.- Leishmania,* L-leishmaniasis

Infections caused by species of the *L.braziliensis* complex generally require treatment with pentavalent antimonal drugs. In cases of MCL early diagnosis is essential for effective treatment. Simple CL caused by the *L.mexicana* complex does not necessarily require treatment. For *L.braziliensis* infections, sodium-stibogluconate is the drug of choice. Relapses are not uncommon and require a second course of treatment, possibly also with another drug (eg. Amphotericin B). In contrast to *L.braziliensis* infections, *L.mexicana* infections respond only poorly to antimonal compounds, but do instead respond well to ketoconazol. Reversely, ketoconazol is not efficient for the cure of *L.braziliensis* infections (Navin *et al*., 1992).

For the treatment of kala azar, pentavalent antimonial drugs have been the therapy of choice since 1916. Due to its toxicity the treatment has to be monitored carefully. Heart and liver are typically affected (Hepburn, 2000). Resistance against pentavalent antimonals has become a serious problem, especially in India. As an alternative drug Amphotericin-B has proved to be effective too. Less toxic lipid-associated formulations of Amphotericin can be administered as well. The combinations of Paromomycin (Aminosidine) with Sb and interferon-γ with Sb are also efficient and may help to reduce side effects of Sb compounds (less Sb is required) (reviewed by Berman, 1997).

The treatment of VL and MCL requires hospitalization for several weeks and the drugs are expensive. New hope for an oral treatment came with the discovery of Miltefosine, which appears to be highly efficient, less toxic, and can be administered orally. If clinical trials are successful, this drug will be very beneficial, especially in India and Sudan (Herwaldt, 1999 b).

1.1.7. Transmission:

The presence of leishmaniasis depends on a variety of ecological and biological factors. Since the various *Leishmania* species depend as much on specific reservoir as on specific vector species, a *Leishmania* focus can only exist if suitable ecological conditions are present for both the host animal species and the sandfly species. The topography and the climate are essential for the maintenance of the life cycle. Only if the reservoir and the vector live close enough together is the transmission of the parasite possible and the infectious cycle maintained.

CL:

CL occurs either as a zoonotic or an anthroponotic infection. Zoonotic CL is caused by *L.major,* with rodents serving as the reservoir. These rodents live usually in colonies and are commonly found in vast uninhabited areas. The rodent burrows provide excellent breeding places for the sandflies. Through the coexistence of rodents and sandflies in the same habitat the natural transmission cycle of *L.major* is maintained. In zoonotic leishmaniasis humans are only

accidental hosts. New endemic foci of CL can emerge when developmental changes take place (eg. building, agriculture). In the Middle East and North Africa the fat sand rat (*Psammomys obesus*) is the main reservoir. Also jirds (*Meriones* sp.) were found to host the parasite (Schlein *et al*., 1982; 1984). In Central Asia, Iran and Afghanistan the great gerbil (*Rhombomys opimus*) was identified as being the reservoir of *L.major* (Strelkova, 1996; Yaghoobi-Ershadi and Javadian, 1996). This gerbil species also hosts nonpathogenic species of *Leishmania* (*L.gerbilli* and *L.turanica*). *L.major* is transmitted predominantly by sandflies of the species *Ph.papatasi*.

Anthroponotic CL has been attributed to *L.tropica*. Major urban centers of the Middle East (Aleppo, Damascus, Baghdad, Tashkent, Teheran and Kabul) were known to be highly endemic for *L.tropica*. Humans were the only known reservoir. The infections were so numerous as to maintain the transmission cycle. In recent years it became increasingly obvious that the classical transmission pattern of *L.tropica* infections has changed. Epidemics in densely populated areas became less common. A shift from an urban to a rural distribution took place. In some of the mentioned Middle Eastern cities, CL has been quite efficiently eradicated by systematic and repeated spraying of houses with insecticides and also as a result of spraying within anti-malaria campaigns. In the past decade the outskirts of cities and villages have been affected predominantly, and infections have occurred more sporadically. These characteristics suggest the existence of an animal reservoir and a zoonotic transmission of *L.tropica*. Until today the animal reservoir of *L.tropica* has not been identified, but several animal species are suspected to function as reservoir. *L.tropica* has been isolated from a hyrax (*Procavia* ssp.) in Kenya (Sang *et al*., 1992), from dogs in Morocco (Dereure, 1991) and from a rat (Aljeboori and Evans, 1980). Hyraxes are the proven reservoir of *L.aethiopica* in Ethiopia (Ashford *et al.*, 1973). In Jordan hyraxes are abundant in endemic areas of *L.tropica* and are therefore suspected to be the reservoir (Saliba *et al*., 1997). This has been discussed also by Ashford, (2000). *L.tropica* is predominantly transmitted by sandflies of the species *Ph.sergenti.*

VL:

VL occurs either in a typical zoonotic or an anthroponotic pattern, depending on the species involved. *L.donovani* depends on interhuman transmission (Thakur, 2000). It occurs in epidemics in densely populated areas of India (Bengal, Bihar) and in Sudan. Subclinical infections are thought to exist at such a high number that this alone would be sufficient to maintain the infectious cycle. Besides, PKDL patients are suspected as serving as a reservoir, especially bridging long-term intervals between epidemics. The homophilic nature of the vector

of Indian kala azar, *Ph.argentipes*, supports the hypothesis that humans are the only reservoir in India.

In other regions of the World (Mediteranean countries, the Middle East, Central Asia and China) VL is a zoonosis. The causative agent is *L.d.infantum,* which is transmitted by phlebotomine sandflies mainly of the subgenus *Larroussius*. Canids, predominantly dogs, serve as a reservoir (Adler, 1962). Dogs suffer from canine visceral leishmaniasis (CVL), a disease closely related to human VL. In addition, dogs suffer typically from various dermal symptoms. The distribution of the disease is typically rural. Wild canids (eg. foxes and jackals) seem to be an important factor for the distribution of the parasite over larger geographical areas (Baneth *et al*., 1998). The epidemiology of *L.d.chagasi* in the New World is very similar, causing VL in humans and CVL in canids.

MCL:

CL and MCL of the New World are zoonotic, with small forest rodents and sloths serving as reservoir. Forest workers, hunters and settlers on cleared forest land are especially at risk. Sandfly species of the genus *Lutzomyia* and *Psychodopogus* are the vectors.

1.1.8. Antiepidemic measures:

Control measures target the interruption of the transmission cycle. For efficient control, the ecology and epidemiology of the disease have to be understood (differences depending on species and local ecology). Depending on the circumstances (eg. zoonotic or anthroponotic transmission), the control of either the reservoir or the vector is advisable. The human link in the transmission cycle can be controlled by taking personal precautions.

Prevention of human infections:

As sandflies are only active at night, inhabitants of endemic areas or travellers in endemic areas can diminish the risk of exposure to sandfly bites by long sleeve clothing during evening hours, application of insect repellents and usage of bed nets. Fine mesh screens for windows are advisable as well. In the case of anthroponotic leishmaniasis, early case detection and treatment are the most important control measures. Despite various attempts to develop a vaccine no efficient immunization is yet available against leishmaniasis (reviewed by Eisenberger and Jaffe, 1997).

Control of the reservoir:

One effective measure for the control of zoonotic CL is the deep ploughing of the area around houses, in order to destroy the rodent burrows. In the northern Jordan Valley CL cases became rare in recent years due to extensive agriculture and development which had resulted in the destruction of the natural habitat of the *Psammomys* (personal communication with Dr. Alon Warburg). In order to control VL, different measures have been employed. It has been shown in a study in Brazil that the removal of infected dogs led to a lower incidence but did not eradicate the disease (Ashford *et al*., 1998). Vaccination of dogs might be an alternative approach for the future (Tesh, 1995). Another control measure has been presented by Killick-Kendrick *et al*., (1997), who found that deltamethrin impregnated collars protect dogs very efficiently from sandfly bites; biting activity was reduced by 96%.

Vector control:

One important goal is the destruction of breeding places. This involves the closure of cracks in walls and the removal of rubble. A common measure is the spraying of houses with insecticides (Alexander *et al*., 1995; Tayeh *et al*., 1997 a). Biological control measures seem to be effective as well (Robert *et al*., 1997). In India kala azar had been almost eradicated after an anti-malaria campaign. Since the spraying of houses has been stopped, the disease has returned.

1.1.9. Leishmaniasis in the Middle East:

The Middle East is endemic for CL and VL. In most countries of the Middle East the three Old World species, *L.major*, *L.tropica* and *L.d.infantum* are prevalent. Even though urban *L.tropica* infections became less frequent the overall picture is that CL and VL are emerging diseases in the region. Several new outbreaks were reported during the last decade. Besides developmental changes, the political instability is an important factor: non-immune population groups as military troops, workers and also refugees enter endemic areas. More than 50 nations provide peacekeeping forces in the Middle East. In some of the home countries of these multinational forces, leishmaniasis has never been diagnosed before and therefore might be misdiagnosed. An endemic focus of *L.major* emerged in the northeastern Sinai among the multinational peace-keeping forces stationed in this area (Fryauff *et al.* 1993). Norton *et al*., (1992) reported about 23 Fijian members of an international observer force who had acquired CL. Visceral infections caused by *L.tropica* have been reported from veterans of Operation Desert Storm (Magill *et al.,* 1993).

In recent years *L.tropica* infections have been reported in rural areas in the Middle East and North Africa. Either new endemic foci or a higher incidence in already known endemic areas

were reported in Jordan (Saliba *et al*. 1993; 1997), Morocco (Dereure *et al.,* 1991), Oman (Scrimgeour *et al.,* 1999), an Afghan refugee camp in Pakistan (Rowland *et al.,* 1999) and in Aleppo in Syria (Ashford *et al*., 1993; Tayeh *et al*., 1997 b).

L.major, L.tropica and *L.donovani* ssp. have been reported in Iraq, Iran and Saudi Arabia (Aljeboori and Evans, 1980; Hatam *et. al*., 1997; Momeni and Aminjavaheri, 1994; Peters *et. al*., 1985). VL has been reported in Jordan (Qubain *et al*., 1997), in Tunisia as an emerging disease (Ben Salah *et al*., 2000), and new foci were reported in Libya (Mehabresh, 1994). *L.donovani infantum* has been reported in Lebanon and Tunisia (Nuwayri-Salti *et. al*., 1994; Gramiccia *et al*., 1991) causing CL. In Iran, CL is endemic in many parts of the country. *L.major* and *L.tropica* are prevalent, partly in the same regions. In 1989, 15,757 cases of CL were reported, in some areas up to 70% of the population have scars from past infections, the incidence being 1.4/1,000 in the Isfahan province of Iran (Momeni and Aminjavaheri, 1994). The disease is common in the cities as well as in the rural areas. Leishmaniasis is endemic also in Turkey (Uzun *et al*., 1999).

Leishmaniasis in Israel and the West Bank:

CL has first been reported in the area of Jericho in the beginning of the 20th century (Huntemueller, 1914; Canaan, 1916). Settling and development of arid and semiarid areas has taken place over the last few decades, which gave rise to the emergence of new foci of CL (see map, Figure 4). According to reports from residents of Jericho, the disease was not seen in the years before the Six Days War in June 1967. This was due to spraying against malaria with DDT. A survey of Israeli soldiers who had been in the greater Jericho area during the war, revealed that the disease was still hyperendemic, with an incidence of 50% during an average exposure time of 1 month (Naggan *et al*., 1970). The recent epidemiology of CL in the Jericho district has been profoundly studied by Jawabreh (2000). The epidemiology of leishmaniasis in Israel has been reviewed by Greenblatt *et al.*, (1985). *Leishmania major* accounts for the majority of infections with *Leishmania* in the country. It is endemic in the Jordan Valley, the Jericho area, along the Dead Sea, in the Arava and the Negev. CL caused by *L.major* has been thoroughly studied over many years, and the reservoir animal species (*P.obesus, M.crassus*) as well as the vector species (*Ph.papatasi*) have been identified (Schlein *et al.,* 1982; 1984).

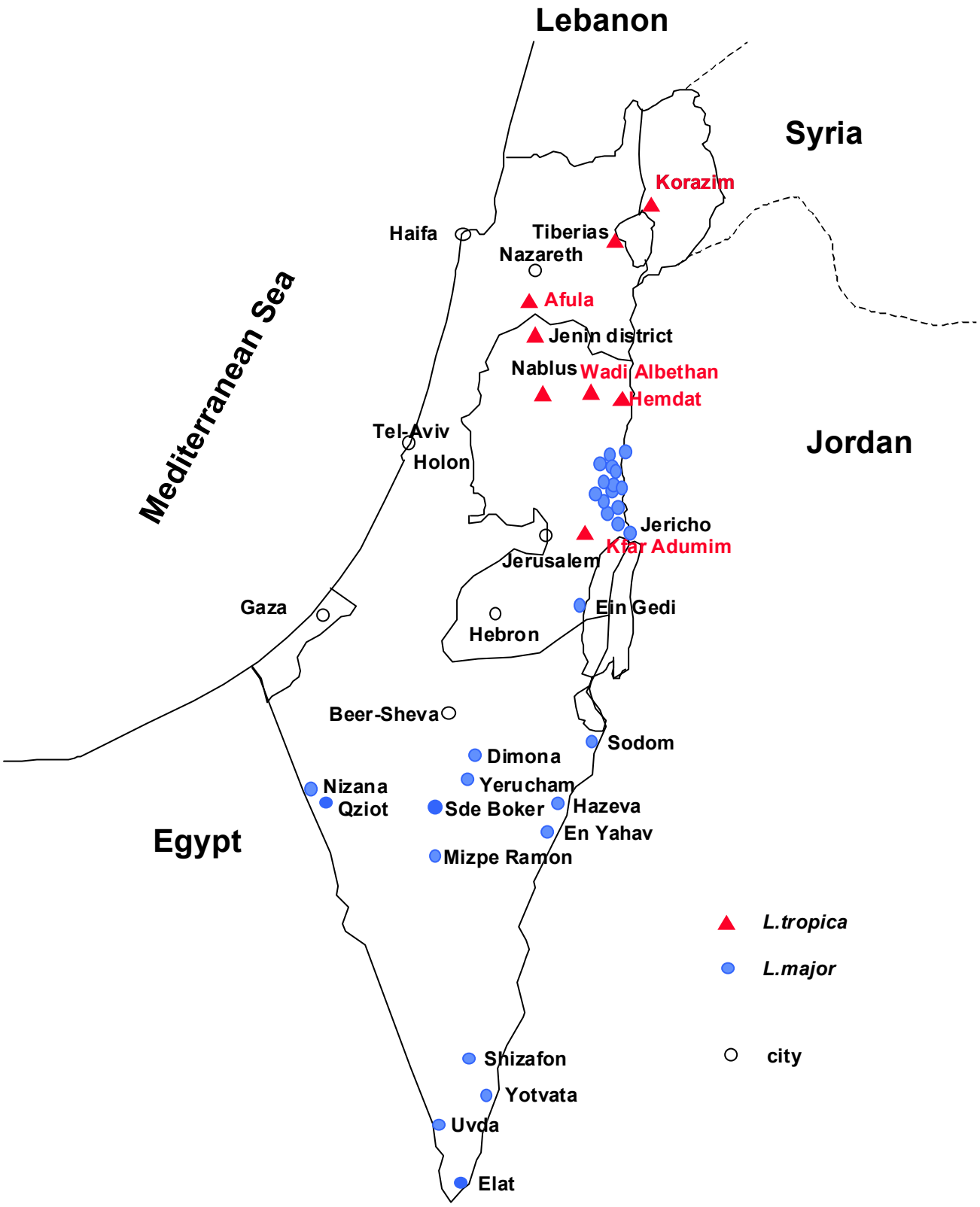
Leishmania tropica is endemic in a number of semiarid hilly areas in central and northern Israel as well as in the northern West Bank (Samaria), the Jenin district being a major endemic area (Abdeen *et al*., 2000, in press). An outbreak has been reported in Salfit (Blum, 1978), an Israeli settlement north of Nablus. The species has never been finally confirmed, but the hilly environment was highly suggestive of a focus of *L.tropica*. Another *L.tropica* focus has been

reported by Klaus *et al.,* (1994): since 1989, CL had been diagnosed in 19 residents of Kfar Adumim, a village between Jerusalem and the Dead Sea. In 6 patients the cultivation of promastigotes succeeded and *L.tropica* was identified. Oren *et al.*, (1991) reported about a 21 year old Israeli suffering from VL caused by a *L.tropica* variant who came from the area south of the Sea of Galilee. Hyraxes were found to be abundant in the environment of several *L.tropica* foci. They are highly suspected as being the reservoir. According to Sawalha (2001) more than 700 cases of CL have been reported from many single foci in the northern West Bank over the last 10 years. The causative species has not been identified, but the geographical area suggests *L.tropica.*

VL caused by *L.d.infantum* is an emerging disease in the northern West Bank. More than one hundred (127) human VL cases have been recorded over the past 10 years. Most cases were reported in the Jenin district, followed by Hebron, Tulkarem and Ramallah. Predominantly children between 1 and 6 years of age were affected (Abdeen *et al*., 2000, in press, Greenblatt *et al.*, 1985, Qubain *et al.*, 1997). A serological survey in an Arab village in an endemic area of VL in western Galilee showed a prevalence of 10% (Ephros *et al*., 1994). CVL has been diagnosed in dogs in central Israel. In a village close to Tiberias (Wadi Hamam), 4% of the dogs were seropositive and *L.donovani sensu lato* was identified from cultured parasites (Jaffe *et al*., 1988). Wild canids are suspected of playing a major role in distribution of the disease over greater distances. More than 5% of the foxes and jackals have been found to be positive by serology. In two villages west of Jerusalem the prevalence was about 10% of the domestic dog population (Baneth *et al*., 1998). Some dogs showed severe signs of CVL. In the northern West Bank, where human VL is endemic, the *L.tropica* foci are adjacent or even overlapping.

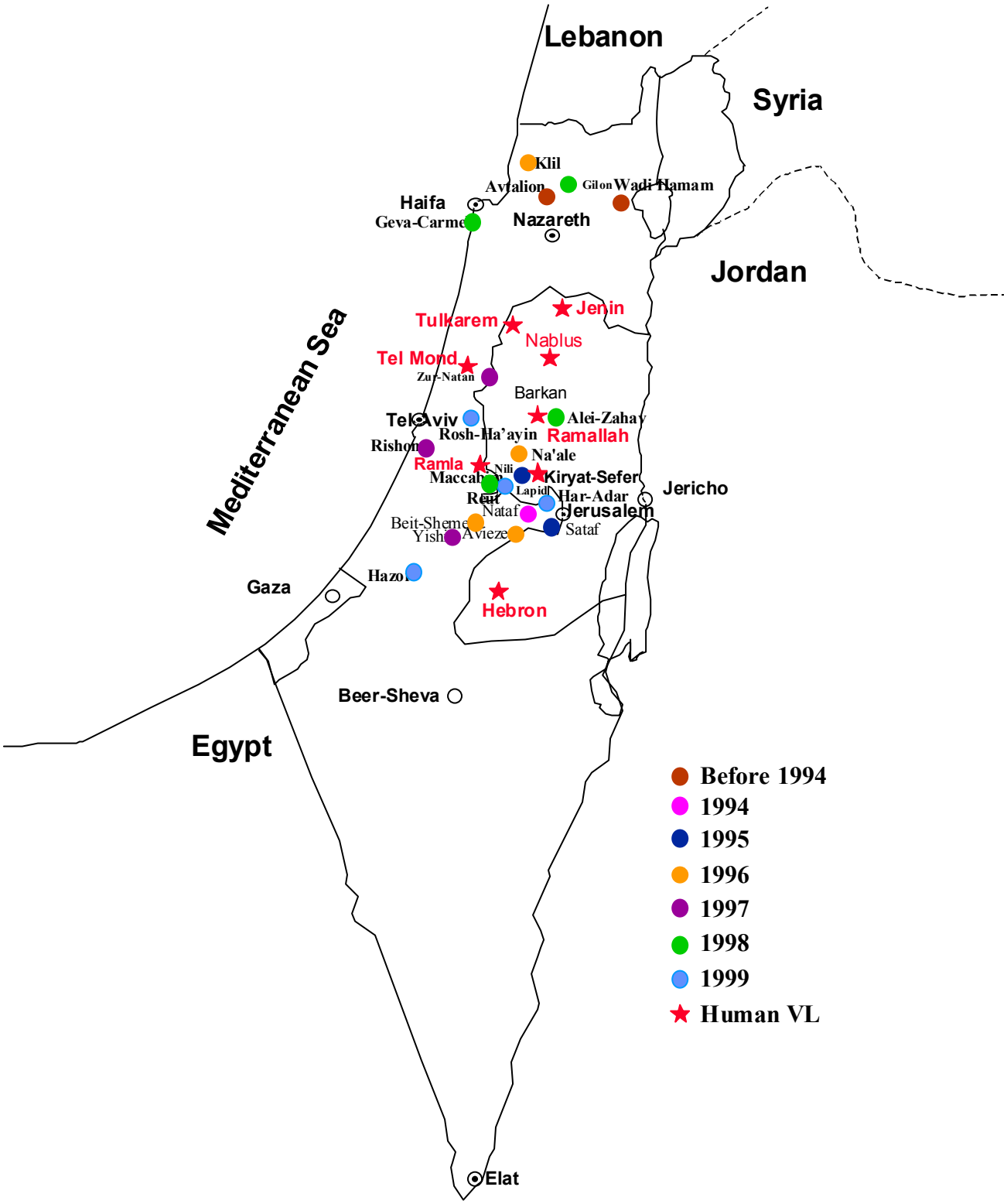
Leishmaniasis is by law a reportable disease in Israel, but nevertheless the disease seems to be greatly underreported. The number of cases reported to the Israeli Ministry of Health over the last few years did not exceed the number of patients seen in the Hadassah Hospital, which is only one of several hospitals where patients with CL are treated. The Bedouins of the Negev and the Judean Desert are familiar with the disease. Since the disease is well-known among the inhabitants in endemic areas, simple cutaneous lesions are often diagnosed by the patients themselves and are left for self cure. As many young Israelis tour Central and South America after military service, New World leishmaniasis is increasingly diagnosed among returners (Zlotogorski, 1998).
Figures 4 and 5 show the distribution of CL and VL over the last decade (1990-2000) in Israel and the West Bank.

Figure 4: Cutaneous Leishmaniasis in Israel and the West Bank



This map is based on the data of CL patients who visited the Dermatology clinic of the Hadassah Hospital since 1990 and on information collected during the study period. The information, especially on the West Bank, is not complete.

Figure 5: Canine Visceral Leishmaniasis in Israel and the West Bank 1988-1999



This map was created by Gad Baneth, School of Veterinary Medicine, Hebrew University, Rehovot.

1.2. Review of the diagnostic methods for leishmaniasis:

1.2.1 Direct identification by classical routine methods:

Microscopy:

Classically the diagnosis of leishmaniasis relies on direct microscopical identification of amastigotes. Its sensitivity is limited to about 60% (Rodriguez *et al*., 1994), which has been found by many others too. No species-specific diagnosis can be achieved. Smears from skin lesions, tissue biopsies or aspirates from bone marrow, spleen or lymph nodes are used. Microscopy is performed on Giemsa stained smears or sections of the named specimens. Typically round to oval amastigotes are seen inside of macrophages. Sometimes they are also found extracellular. The densely stained (violet) rod-shaped kinetoplast should be always identified. Biopsies have shown a greater sensitivity than smears for the diagnosis of CL (Rodriguez *et al*., 1994). Andresen *et al*., (1996) identified amastigotes in 76% of histological sections, but only in 55% of smears taken from CL lesions. To improve the microscopical sensitivity of smears it has been recommended that a superficial slit be made radially into the lesion and that some tissue be scraped off with a surgical blade (Herwaldt, 1999 a; Hepburn 2000). This collection method is much less invasive than taking biopsies. For the microscopical diagnosis of VL, splenic aspirates proved to have the highest detection rate (96.4%, 84 out of 88 patients), followed by bone marrow aspiration (70.2%) and lymph node aspiration (58.3%) as shown by Zijlstra *et al*., (1992) and also reported by others. Bleeding complications are feared, but are low (0.6%), if applied exclusively to patients with coagulation parameters within the normal range. In peripheral blood the detection rate by microscopy is very low.

Culturing:

Cultures are obtained from aspirates or biopsies from the above-named sources. Cultures are usually grown in NNN (Nicolle-Novy-McNeal) and in Schneider's Drosophila medium. Culturing of promastigotes is required for a number of diagnostic methods such as isoenzyme analysis, and some of the serological and DNA-based methods. The sensitivity of culturing is variable and depends on various factors, as for example the viability of collected parasites, the strain, the media (different requirements for different species), the presence of a superinfection, and the expertise of the investigator. Different success rates have been reported, ranging between 4% in Nicaragua (6 of 143) (Belli *et al*., 1999) and 95% (61 of 64) in Switzerland (Grimm *et al*., 1996). Mostly a sensitivity of 40-50% was found (Rodriguez *et al*., 1994). Culturing is expensive and time-consuming as cultures sometimes become positive only after two or more weeks (Gramiccia *et al.,* 1991). It depends on adequate facilities, which are often not available in less-developed countries. Furthermore, cultures are susceptible to contamination. In cases of double

infections one strain usually outgrows the other, so that misdiagnosis can occur (Klaus *et al*., 1994).

1.2.2. Indirect diagnosis:

1.2.2.1. Cellular immunity:

Leishmanin skin test (LST) or Montenegro skin test:

The LST is widely used for the screening of the exposure to *Leishmania* parasites within endemic areas. Analogous to the tuberculin test, leishmanial antigen (killed promastigotes) is applied intradermally and a delayed hypersensitivity reaction is measured after 48-72 h. A reaction is seen in people with previous contact to the antigen who have developed cellular immunity. Conversion occurs after several weeks in CL, and in VL usually only after treatment and cure (Peters and Killick-Kendrick, 1987). Present and past infections can not be differentiated.

Lymphocyte proliferation assay (LPA):

Alternatively a lymphocyte proliferation test can be used to measure the cellular response (Alvarado *et al.* 1989). In contrast to the LST one patient contact is sufficient, which may facilitate larger field studies. On the other hand the LPA is more labour intensive.

1.2.2.2. Serological methods:

Serology is suitable for mass screening within epidemiological surveys on VL or CVL. It is limited in its use for the diagnosis of leishmaniasis since present, subclinical and past infections generally not be discriminated. It is not reliable in CL since antibodies are often not detectable (Herwaldt, 1999). Cross reactions often complicate the interpretation (Wilson, 1995). Another disadvantage of serology is the fact that it is generally not reliable in immuno-compromised patients (Pizzuto *et al*., 2001; Agostoni *et al*., 1998).

Enzyme linked immunosorbent assay (ELISA):

The ELISA technique has been adapted especially to the diagnosis of VL by Hommel *et al*., (1978). Monoclonal Antibodies have been developed and are widely used for the ELISA, Western Blot and immunofluorescence assays (Jaffe and McMahon-Pratt, 1983; 1987; Jaffe *et al.,* 1984; Jaffe and Sarfstein, 1987; Baneth *et al*., 1998). A highly specific and sensitive ELISA was developed by Jaffe and McMahon-Pratt (1987) for the diagnosis of VL. The assay was based on specific antibodies competing with serum antibodies. This competitive assay proved to be superior to direct binding tests, since no cross reactivity was reported, but it is not generally

used. In a comparative study on different serological methods conducted on 49 patient sera from India, the ELISA using a recombinant antigen proved to be more sensitive (100%) than the regular ELISA (77%), and was more sensitive than the DAT (90%) (Raj *et al*., 1999).

Immunofluorescence assay (IFA):

Immunofluorescence assays can be employed to detect either antibodies in patient sera or to identify the antigen. The IFA has been adapted to detection of *Leishmania* antibodies in patient sera by Walton *et al*., (1972). Rachamim *et al*., (1991) found in a comparative study in dog sera that the ELISA yields the same qualitative diagnosis as the IFA. It was concluded that the ELISA can replace the IFA due to its better practicability and its advantage in examining many more sera simultaneously. Mancianti *et al*., (1995) found a slightly higher sensitivity with the ELISA (99.5%) than with the IFA (98.4%), but the IFA was more specific (100%) than the ELISA (97.1%). The study was conducted with 290 dogs (186 *Leishmania* infected and 104 control animals).

Direct agglutination test (DAT):

The DAT is a comparatively simple and reliable screening test suitable for field work. It is based on the agglutination of positive sera with stained promastigotes and can be performed in microtiter plates (Allain and Kagan, 1975; Harith *et al.,* 1986; Zijlstra *et al*., 1991; 1992). In a study on treated VL patients the DAT was reported to be to 100% sensitive and to 99.3% specific. Oskam *et al*., (1999) used a freeze-dried antigen which is more stable and therefore improves the usefulnes of the DAT for field studies.

Western Blot:

The Western Blot relies on the recognition of *Leishmania* antigens by anti-leishmanial antibodies. Antigenic proteins of the parasites are separated by electrophoresis and are incubated with patient sera. In the Western Blot several different antigenic products can be recognized by different antibodies simultaneously in one assay. It was found that the sera of PKDL patients consistently recognize a specific antigen which is not recognized in VL patients without PKDL. This could be utilized for the identification of PKDL patients (Salotra *et al*., 1999).

A recent improvement in serological diagnosis is the development of the rK39 dipstick test. One antigen (rK39) with high antigenicity has been selected and is recognized by the serum of kala azar patients. It is successfully employed in the diagnosis of VL in Nepal. It was found to be 100% sensitive and 100% specific when tested in 14 newly diagnosed VL patients and 113

controls (Bern *et al*., 2000). It is comparatively inexpensive and results are obtained within minutes.

1.2.3. Identification of *Leishmania* species and subspecies:

Isoenzyme analysis:

The analysis of isoenzymes is a generally accepted reference method for the species-specific diagnosis of *Leishmania* strains. The electrophoretic mobility of soluble enzymes is examined on starch or cellulose acetate gels. Patterns of single or multiple bands are seen. A consistent profile of isoenzyme patterns is found in closely related strains, which is also referred to as zymodemes. Between 10-20 enzymes have to be examined in order to classify new strains into their zymodemes (Miles *et al*., 1980; Aljeboori and Evans, 1980; Peters *et al*., 1985; Oren *et al.*, 1991; Barral *et al*., 1991; Kreutzer *et al*., 1993). Gramiccia *et al*., (1992) found that viscerotropic and dermatotropic *L.d.infantum* strains differed in their zymodemes. Isoenzyme analysis is work-intensive, time-consuming and not feasible for a regular laboratory. It is usually performed in specialized laboratories which are also internationally acknowledged to label new strains with codes according to their zymodemes (eg. LON- for London School of Hygiene and Tropical Medicine, or MON- for University of Montpellier).

Excreted Factor (EF):

This method allows species specific diagnosis of *Leishmania* strains (some species can be distinguished, some not). The EF refers to soluble antigenic substances (glycoconjugate compounds) which are present in the medium of growing promastigotes. Culture media is used in a gel diffusion test in which the EF compounds are precipitated by antileishmanial antibodies of the same species (Schnur *et al*., 1972).

1.2.4. DNA based methods:

1.2.4.1. Classical methods: hybridization and restriction:

Efforts have been made to develop hybridization methods. Before the introduction of PCR, Southern Blotting, using radiolabelled probes for hybridization, was one of the most sensitive detection methods. Smith *et al.,* (1989) were able to routinely detect 10,000 promastigotes. Washed promastigotes were blotted on nitrocellulose filters and hybridized with radiolabelled, cloned fragments of minicircles (das Gupta *et al*., 1991). Gramiccia *et al*., (1992) have employed a kinetoplast probe specific for *L.d. infantum*. Laskay *et al.*, (1991) introduced a dot blot hybridization method using squashed sandflies on nylon membranes and *L.aethiopica* DNA as a probe. The hybridization detected every infected sandfly and reached the same level of

sensitivity as microscopy on infected sandflies. A weak signal was obtained from a minimum of 100 promastigotes. Lambson *et al.*, (2000) developed a probe based on a cloned fragment of a minicircle which hybridized with DNA from the *L.donovani* complex only. Dot blots of promastigotes with this radiolabelled kDNA probe confirmed that the detection limit ranges between 10^3 –10^4 parasites, as others had shown previously.

Also the digestion of purified kDNA with analysis of the restricted fragments (RFLP-analysis) was used for diagnosis (Jackson *et al.,* 1984). The sensitivity of these methods is not sufficient for direct diagnosis in clinical material.

1.2.4.2. Modern methods based on the polymerase chain reaction (PCR):

The polymerase chain reaction (PCR) provides an excellent tool for the diagnosis and characterization of various infectious agents. It proved to be very useful for the diagnosis of leishmaniasis too. The method is based on the enzymatic amplification of selected DNA sequences, which are made visible by gel electrophoresis. PCR has a high potential for sensitivity, up to the detection of single parasites (Rodgers *et al*., 1990; Lopez *et al*., 1993; Noyes *et al*., 1998; Harris *et al*., 1998). In many studies PCR was found to be more sensitive than microscopy (Osman *et al*., 1997 a), and it proved to be more sensitive (100%) when compared to serology (63%) (Ashford *et al*., 1995). So far, several different PCR approaches have been developed for the diagnosis of leishmaniasis.

Depending on the objective, different targets for amplification are chosen: for diagnosis multi-copy sequence repeats are usually selected, implying the potential for high sensitivity. For this purpose the sequences need to be highly conserved either within the genus or within the species, depending on the specific aim. For genetic characterization of individual strains, more variable regions are selected, which usually do not appear in high copy numbers and are therefore less sensitive targets. Culturing of promastigotes is usually required for genetic characterization. Sensitivity and specificity can be enhanced by the hybridization of PCR products (southern blotting). The specificity can be improved by restriction fragment analysis of the PCR-amplificates.

Targets for diagnostic PCR: Kinetoplast DNA (kDNA)

The targets with the highest copy number (10,000) are the kDNA minicircles. The minicircles have a size of about 800 bp with specific differences in sizes among some of the *Leishmania* species. They divide into subclasses of different sequences, but all of them share the same conserved sequence of 120 bp. This fact has been utilised for a genus-specific diagnostic PCR approach introduced by Rodgers *et al*., (1990) and later employed by others (Rodriguez *et al.,*

1994; Ashford *et al.,* 1995; Laskay *et al.,* 1995*;* Reale *et al.,* 1999). The major part of the minicircles consists of variable sequences. The different subclasses show variations, which to some extent are shared among species and to another are even different among clones of the same strain. This can be explained by a fast rate of sequence evolution of the minicircles. In contrast, some minicircle classes are well preserved within the species, even from geographically distant origins. Gutiérrez-Solar *et al.,* (1995) showed that minicircle sequences from a well preserved minicircle class can be almost identical, even if the strains are isolated in China or in Europe. These facts make the design of species-specific oligonucleotides and probes problematic. Nevertheless, several approaches were undertaken, which have proved to be useful for species-specific and comparatively sensitive diagnosis. One pair of oligonucleotides was introduced by Smyth *et al*., (1992) for the diagnosis of kala-azar patients, which amplified whole minicircles of different species. Bhattacharyya *et al*., (1996) developed genus specific kDNA primers able to amplify whole minicircles. Eresh *et al.,* (1993) introduced primers with the purpose of amplifying the species from the Old World and specifically to differentiate between *L.major* and *L.tropica*. These oligonucleotides were applied to purified DNA, but the sensitivity has never been studied (Klaus *et al*., 1994).

2.Nuclear targets:

Two genomic targets have been preferably selected: The small subunit ribosomal RNA (ssu rRNA) and the spliced leader sequence or mini-exon gene. Both targets are present in about 200 copies. Due to the fact that these gene repeats consist of both conserved and variable regions, which are often species-specific, these targets are suitable for the development of more specific diagnostic approaches. The ssu rRNA gene has been successfully used as a target (van Eys *et al*., 1992; Campino *et al*., 2000). The internal transcribed spacer (ITS) within the ribosomal operons (ssurRNA) is another target which has been selected by Guevara *et al*., (1992), Cupolillo *et al*., (1995) Schönian *et al*., (2000) and El Tai *et al*., (2000). The latter showed that the PCR was highly sensitive using clinical material. Combined with restriction fragment analysis of the amplified ITS region all species complexes could be identified. Also the mini-exon gene was successfully used as a target (Fernandes *et al*., 1994, Ramos *et al*., 1996; Harris *et al*, 1998). Harris *et al*, (1998) developed a multiplex PCR which was able to discriminate between the New World complexes of *Leishmania* and which was also very sensitive.

PCR and hybridization:

In order to enhance the sensitivity and also to confirm the specificity, PCR has been combined with hybridization. In recent years non-radioactive detection methods have been introduced.

Lopez *et al.,* (1993) were able to increase the sensitivity of a kDNA based PCR 10 fold by hybridizing the PCR product to membrane-bound oligonucleotides. The PCR product was biotinylated and colorimetrically detected by a streptavidin-alkaline phosphatase (SAP). Rodriguez *et al.,* (1994) developed kDNA probes able to distinguish between *L.mexicana* and *L.braziliensis* infections. The combination of PCR with primers 13A/13B and subsequent hybridization proved to be highly sensitive as well as highly specific. 233 biopsies of skin lesions suggestive of CL were examined. PCR products were seen in 226 (97%) of the patients. After hybridizing the 120 bp product, 117 samples hybridized to a *L.mexicana* specific probe and 109 hybridized to a *L.braziliensis* specific probe. The results reflected the epidemiological situation in Venezuela very well, when the origin of the patients was compared to the results. Furthermore, the results were confirmed by restriction patterns found after digestion with *Msp* I on purified kDNA from 60 positive cultures. Nuzum *et al*., (1995) evaluated the usefulness of PCR on peripheral blood for the diagnosis of kala-azar. Peripheral blood mononuclear cells (PBMC) were separated and lysed. The PCR product was biotinylated and bound to a specific oligonuctleotide probe in a microtiter plate, followed by an enzymatic reaction, which could be detected by an ELISA reader. Laskay *et al.*, (1995) chose a similar approach, combining PCR with primers 13A/13B and Southern Blotting using *L.aethiopica* kDNA as a probe to confirm the specifity. The hybridization enhanced the sensitivity 10 fold so that DNA corresponding to 0.01 promastigote was detectable. Also digoxigenin (DIG) labelled probes were used and hybridization was visualized by a chemiluminescent signal (Rodriguez *et al.,* 1999; Reale *et al*., 1999). The latter found the genus specific kDNA-PCR (13A/13B), to be sensitive up to 0.1 fg of purified DNA (corresponding to 0.01 parasite), when combined with hybridization.

1.2.4.3. Molecular strain typing of *Leishmania:*

For genetic characterization as well as for species-specific diagnosis several other PCR-based methods have been developed:

Randomly amplified polymorphic DNA analysis (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR):

For RAPD/AP-PCR non-specific oligonucleotides are employed, which yield highly polymorphic products (Bhattacharyya *et al*.,1993; Schönian *et al*., 1996). Characteristic patterns for PCR products of different sizes are obtained. Mimori *et al.*, (1998) used single products obtained by the AP-PCR to develop species-specific primers for five *Leishmania* species of the New World. Eisenberger and Jaffe, (1999) employed a *Leishmania*-specific oligonucleotide in combination with a non-specific permissive oligonucleotide. Banding patterns were

characteristic for each species complex, occasionally showing also regional differences within the species. RAPD/AP-PCR-methods are generally not suitable for direct diagnosis: the sensitivity is not sufficient and the conditions need to be highly standardized. The patterns show alterations if the PCR conditions or the amount of template is changed. Due to the characteristics mentioned these methods are limited to the use of purified DNA (Noyes *et al.*, 1996; Eisenberger and Jaffe, 1999).

Restriction fragment length polymorphism (RFLP) or Schizodeme analysis:

Amplified minicircle DNA is restricted enzymatically into fragments, which are visualized by gel electrophoresis. The fragment patterns or schizodemes are specific, either for species or for individual strains. The polymorphism of patterns indicates differences in sequences. Schizodeme analysis is a valuable tool for epidemiology and for species-specific diagnosis, usually on the basis of purified kDNA (Noyes *et al*., 1998; Qubain *et al*., 1997). For *L.d.infantum* it was shown that the known zymodeme groups around the Mediterranean could be subclassified by schizodeme analysis into various other groups (Noyes *et al.*, 1998). Also this method is sensitive to minor changes in the conditions. Apart from that, point mutations may change the pattern due to changed restriction sites. This is desirable for genetic characterization, but for direct diagnosis from clinical material the method is neither sensitive nor consistent enough.

1.3. Objectives of the study:

At the onset of this work, microscopy, serology (ELISA, IFA, EF) and PCR-based on cultured parasites (Eisenberger and Jaffe, 1999) were the standard diagnostic methods for leishmaniasis at the Kuvin Center, which is the most advanced and specialized center for the study of leishmaniasis in Israel and the West Bank. The methods followed in this institute therefore reflect the diagnostic standard of the whole country. No sensitive direct (without culturing and not serological) diagnostic method had been established so far. For the diagnosis of patients as well as for epidemiological studies on reservoir animal species there was need for a field applicable sensitive and specific PCR as a routine method.

The prevalence of three *Leishmania* species in Israel and the West Bank, with overlapping clinical pictures and endemic areas, increasing numbers of patients and unidentified reservoir animal and vector species in some areas made the development of improved laboratory tests an urgent priority. In recent years new *L.tropica* foci are emerging, and *L.d.infantum* has been increasingly identified among canids in central Israel (see maps, Figures 4 and 5). Also the fact that some reservoir animals, eg. dogs, may harbour different species of *Leishmania* emphasizes the need of a directly applicable diagnosis method at the species level (Baneth *et al.,*1998;

Dereure *et al.* 1991). Furthermore, New World CL is a serious problem in the country, as many Israelis travel to Central and South America (Dan *et al*., 1985). There are a number of cases seen annually (~10 in the Tel Hashomer Hospital). These patients need to be diagnosed at least at the complex level, since only infections due to the *L.braziliensis* complex require a three-weeks course of antimonal treatment. Specifically *L.braziliensis* infections need to be distinguished from *L.mexicana* infections. Species of both complexes are prevalent in large parts of Central and South America and have an overlapping geographical distribution and clinical presentation (Eresh *et al.,* 1994). Differential diagnosis within the New World species of *Leishmania* has not been performed in the country to date. Old World infections need to be excluded, since the infections might as well have been contracted in Israel either before or after the journey. Due to immigration from Ethiopia, infections caused by *L.aethiopica* also need to be considered, as a differential diagnosis of other skin diseases. Presumably in no other country is such a variety of causative agents of leishmaniasis seen on a regular basis as in Israel.

Over the last decade many efforts have been made in many parts of the world to improve PCR diagnosis of leishmaniasis. It was emphasized in a recent article on leishmaniasis in the *Lancet* that field applicable, diagnostic methods including species identification are desparately needed (Herwaldt, 1999). Several sensitive PCR methods have been developed by various research groups, using extracted DNA directly from clinical material. It has been shown that clinical material, eg. dermal lesion scrapings (CL) or peripheral blood (VL) could be collected and preserved on filter paper (Osman *et al*., 1997 a; Harris *et al*., 1998; Färnert *et al*., 1999; Campino *et al*., 2000). PCR appeared to be the method of choice, for achieving sensitive and species-specific diagnosis of leishmaniasis in Israel and in the West Bank.

Specific aims:

1. To establish direct PCR diagnosis of leishmaniasis in Israel and the West Bank. This included: testing for the most effective DNA extraction method, developing a sampling strategy (which samples to be collected), optimization of the PCR approach, achievement of the highest possible sensitivity and adaption for routine use.
2. To differentiate between the endemic species *L.major*, *L.tropica* and *L.d.infantum* by direct PCR diagnosis.
3. To establish direct PCR diagnosis of New World leishmaniasis, especially of the *L.braziliensis* complex.
4. To establish PCR methods for the screening of reservoir animals and sandfly vectors.

1. Material and Methods

The polymerase chain reaction (PCR) was selected as the most appropriate method, with the best potential to achieve direct, sensitive and species specific diagnosis. In order to establish the methods, samples had to be collected first. It appeared that dermal scrapings from CL lesions, preserved on filter paper, would be a suitable sampling procedure for this purpose (Harris *et al*., 1998; Färnert *et al*., 1999). Apart from dermal scrapings several other specimens were obtained too, as for example Giemsa stained smears, paraffin embedded biopsies, peripheral blood, aspirates, spleen and liver tissue and also cultures of promastigotes. Samples were collected from patients as well as from various animal species, which were either known or suspected reservoir species of leishmaniasis. The collected and processed samples are presented here as groups. Detailed description of the samples is given together with the results in Tables 5-10.

2.1. Origin of samples:

2.1.1. Reference strains:

Preliminary studies were performed on purified DNA from various strains of all available species. These strains had been collected over many years and were preserved at the WHO-reference center for *Leishmania* strains at the Kuvin Center, Jerusalem. The cultured strains had been characterized previously by EF, isoenzyme analysis and PPIP-PCR (Eisenberger and Jaffe, 1999). The purified DNA of these strains was used for positive controls in every experiment. The New World strains were kindly provided by Linda Oskam, Royal Tropical Institute, Amsterdam. Table 2 shows the reference strains used in this study.

Table 2 : Reference strains

Species	LRC	WHO code	Source	Origin
*L.major**	LRC-137	MHOM/IL/67/Jericho II	human	Jericho
L.major	LRC-465	IPAP/IL/84/UVDA	sandfly	Israel (Uvda)
L.major	LRC-509	MHOM/IL/86/BLUM	human	Jordan Valley
*L.tropica**	LRC-36	MHOM/IQ/66/L75	human	Iraq
L.tropica	LRC-682	MHOM/TR/95/Urfa40	human	Turkey (Urfa)
L.tropica	LRC-747	IL/98/LRC-L747	sandfly	Israel (Kfar Adumim)
*L.donovani**	LRC-133	MHOM/ET/67/HU3	human	Ethiopia
L.donovani	LRC-661	MHOM/SD/??/Khartoum	human	Sudan
L donovani	LRC-751	MHOM/IN/93/BI2302	human	India
*L.d.infantum**		MHOM/TN/80/IPT1	human	Tunisia
L.d.infantum	LRC-716	MCAN/IL/97/LRC-L716	dog	Israel (Nataf)
L.d.infantum	LRC-719	MCAN/IL/97/LRC-L719	dog	Israel (Rishon le Zion)
*L.aethiopica**	LRC-149	MHOM/ET/72/L102	human	Ethiopia
L aethiopica	LRC-495	MHOM/ET/85/Vasa	human	Ethiopia
*L.gerbilli**	LRC-652	MRHO/CN/60/LD3	*Rhombomys opimus*	China
L turanica	LRC-654	MRHO/MN/83/MNR2	*Rhombomys opimus*	Mongolia
L arabica		MPSA/SA/83/JISH220	*Psammomys*	Saudi Arabia

*L killicki**		MHOM/TN/80/LEM 163	human	Tunisia
L.mexicana		MHOM/GT/86/GO22	Oskam/Evans	
L.braziliensis		MHOM/CO/81/MORO	Oskam/Evans	
*L.amazonensis**	WR 669	MHOM/BZ/73/M2269	no information	
*L.guyanensis**	WR 677	MHOM/BR/75/M4147	Oskam/WR	
L.panamensis		MHOM/CR/87/NEL3	Oskam/lab	
L.chagasi		MHOM/BR/74/PP75	LF Schnur	
T.cruzi		-	LF Schnur	
T.lewisi	T22	-	LF Schnur	
L.seymourii	L524	-	LF Schnur	
C.fasciculata	L466	-	LF Schnur	
P.davidii	ATCC 50166	-	LF Schnur	

All strains were part of the WHO Reference Center for *Leishmania* strains at the Kuvin Center. Parts of the collection had been cultivated at the Kuvin Center (coded with LRC- *Leishmania* reference center, animal or human source known). Other strains had been received from others, as for example the internationally used WHO-reference strains (*) (often no information on the source of collection). All strains were identified by isoenzyme electrophoresis, many of them also with additional methods (PCR, EF).

2.1.2. Patients:

Samples were collected from patients visiting the Dermatology clinic of the Hadassah Hospital with lesions suspected of CL. Over a period of more than one year 100 skin scrapings from Hadassah-patients were collected (by Flory Jonas). Skin scrapings and/or cultures were collected from 11 Patients of the Tel Hashomer Hospital, 6 of them had contracted the disease in the New World. Most of the examined patients were Israelis who either live in endemic areas, who had been travelling inside or outside the country or who had been stationed as soldiers in the Judean Desert or in the Negev.

A new focus of CL was discovered in early spring 2000 in a village named Wadi Albethan (West Bank/Samaria), located between Nablus and the Jordan Valley. In 8 households skin scrapings and cultures were collected. Skin scrapings were taken from 23 patients on filter paper and on slides. Eleven lesion aspirates were collected for culturing in NNN and semi-solid media. The number of cultures was smaller since needle aspirations were not performed on facial lesions of infants, and some other individuals refused. Detailed information on single patients is presented in in chapter 3 (Results).

2.1.3. Animal samples:

Desert rodents:

An extensive field study on desert rodents was carried out by Gideon Wasserberg (PhD student of the Department of Life Sciences, Ben-Gurion-University of the Negev). Rodents were trapped in endemic areas of the Negev (Qziot and Nizana), and skin scrapings were collected from the ears for microscopical examination. With the beginning of the cooperation the tissue was collected on sterile filter paper as well (for PCR).

The predominant species was the fat sand rat (*Psammomys obesus*), besides gerbils (*Meriones crassus* and *Gerbil dasyarus*) were trapped and examined. This collection of animal tissue samples provided an excellent study group for testing the reliability of the PCR from tissue preserved on filter paper. DNA extraction and PCR was performed on ear scrapings of 28 individuals of *P.obesus*, 6 *M.crassus* and 19 *G.dasyarus*.

Canids:

Blood of four infected dogs was examined for the presence of leishmanial DNA. A drop of collected peripheral EDTA-blood was preserved on filter paper (received from Dr. Gad Baneth, School of Veterinary Medicine of the Hebrew University, Rehovot). The dogs had been diagnosed previously by microscopy on Giemsa stained lymph node and spleen aspirates.

In an earlier study by the same collegue jackals had been trapped in several areas in central Israel. The jackals had been previously screened for the presence of antibodies against *L.d.infantum* by ELISA and western blotting, partly with borderline positivity. No method for the detection of the antigen had been applied yet. Since PCR was available now it seemed to be worth-while to extract and amplify DNA from tissue of these animals in order to complete the survey. Ears and spleens were examined. The ears of 20 jackals were deep frozen at a temperature of –70°C. The spleens of 14 individuals had been preserved in buffered formalin. This was not very promising for good PCR results but it was worth-while to examine.

Hyrax:

Since hyraxes were highly suspected serving as reservoir for *L.tropica* (Photo 19), it was of interest to examine hyrax tissue. A parched body of a hyrax was found next to a cave in which a sandfly infected with *L.tropica* (LRC-747) had been trapped a few days earlier. The cave was close to Kfar Adumim, an Israeli settlement in the Judean Desert, one of the emerging *L.tropica* foci in the country. Most human cases had occurred in the periphery of the settlement, facing the slope with the caves (Photo 13). The presence of fecal remains of hyraxes as well as the corps indicated that these caves were inhabited by hyraxes. Several specimens of the hyrax were extracted, namely bone powder from the femur, tissue from the skin and nose. Ear tissue was not available. One ear sample from a living hyrax, which had been trapped in the Galilee (by Gunther Müller, Kuvin Center), was examined too.

2.1.4. Cultivation of *Leishmania* promastigotes:

Cultures were obtained by Lionel Schnur (Kuvin Center, Hebrew University) and Flory Jonas (Dermatology Clinic, Hadassah Hospital), or collected during visits (Wadi Albethan, Tel

Hashomer Hospital). One culture derived from a sandfly trapped in a cave close to Kfar Adumim, one of the major *L.tropica* foci in the country (Alon Warburg, Kuvin Center).

2.2. Laboratory work:

2.2.1. Sampling:

The study focused on dermal scrapings preserved on filter paper. Skin scrapings were obtained from patients with cutaneous lesions and from rodents. After desinfection with 70% ethanol a small incision was cut with a disposable surgical blade radially into the lesion, and some tissue was scraped off as described by Herwaldt, (1999). It was important to obtain tissue with as little blood as possible. The material was blotted on UV-radiated and autoclaved 3 mm filter paper (Schleicher and Schüll, Germany) and on glass slides for Giemsa staining. The tissue samples were wrapped with aluminium foil and stored at room temperature. Prior to extraction, the scraped tissue spots were cut off of the filter paper using sterile surgical blades. A new blade was used for each sample. The tissue samples were transferred to screw-cap 1.5 ml tubes containing either 500 µl NET buffer, 500 µl guanidine thiocyanate solution or 250 µl chelex (5%), depending on the extraction method used.

2.2.2. Giemsa staining of smears:

Smears were collected on glass slides, air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). After 20 minutes of staining the slides were washed with tap water and air dried. The stained smears were examined under the microscope with a 40x lens and with a 100x oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive kinetoplast was found the smear was declared positive. When no amastigotes were seen after 15 minutes the smear was declared negative. Microscopy of the smears of the Hadassah patients and of the desert rodents was performed by Flory Jonas and Gideon Wasserberg respectively. Many of the patient smears and some of the rodent smears were double checked, the observations were in concordance. All samples collected during visits (Wadi Albethan, Tel Hashomer Hospital) were stained and examined in our laboratory.

2.2.3. Collection and cultivation of *Leishmania* strains:

Cultures were obtained by needle aspiration from the border of the lesions. Usually it was necessary to inject about 0.1 ml of sterile 0.9% saline into the lesion in order to aspirate a drop of fluid as described by Herwaldt, (1999). The aspirated fluid was discharged into two culture tubes, one containing semisolid (SS) blood agar (Schnur and Jacobsen, 1987) and the other NNN

medium (Novy-MacNeal-Nicolle medium) overlayed with Schneider's (Gibco, Grand Island, NY; Biological Industries Beit Haemek, Israel) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 200 µg/ml streptomycin, and 200 U/ml penicillin. The cultures were incubated at 24-28°C. After a few days one drop of media was examined under the microscope. If promastigotes were found the cultures were transferred into Schneider's *Drosophila* medium for further culturing. The parasites were cultivated in 5 ml of Schneider's medium for 2-3 days before they were transferred to a larger volume (40 ml). Negative cultures were examined again several times and in case of no growth they were discarded only after 3 weeks. DNA-extraction was performed from 40 ml of densely grown parasites (see later).

2.2.4. DNA extraction:

Several different methods were tested, in order to find reliable and simple methods which could be adopted in a clinical laboratory and also be employed for epidemiological studies (high number of samples).

Phenol-chloroform-extraction:

1. Extraction from clinical samples, mostly dermal scrapings preserved on filter paper:

Each tissue sample was transferred into a 1.5 ml tube and lysed with 500 µl of NET buffer (50 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl). Sodium dodecyl sulfat (SDS) was added to a final concentration of 5 %. RNAse was added to a final concentration of 100 µg/ml (5 µl of a 10 mg/ml solution) and the samples were incubated at 37°C for 30 minutes. Proteinase K (10 µl of a 10 mg/ml solution) was added to a final concentration of 200 µg/ml. The samples were digested over night, rotating in a hybridization oven at 60°C. Equal volumes of saturated phenol were added to each sample (500 µl). The samples were mixed gently for 5 minutes. After centrifugation at high speed (12000 rpm) for 5 minutes at room temperature the upper phase was carefully removed and transferred to new 1.5 ml tubes. A phenol and chloroform mix (chloroform/isoamylalcohol 25:1) was added in equal volumes to each sample (250+250 µl). The samples were mixed gently and centrifuged for 3 minutes. The upper phase was again transferred to a new tube. Equal volumes of chloroform were added and the samples were centrifuged for 1 minute. The upper phase was once again transferred. The DNA samples were precipitated with 50 µl of 3M sodium actetate (NaAc 300 mM, pH 6.0) and one volume (500 µl) of cold (-20°C) isopropanol and then stored on ice for 30 minutes. Subsequently, the samples were centrifuged at 14,000 rpm for 45 minutes at 4°C. The isopropanol was removed and the DNA pellets were washed with cold (-20°C) 70% ethanol (250 µl). The samples were centrifuged for 10 minutes.

The ethanol was removed, and the DNA pellets were vacuum dried. The DNA samples were then resuspended in 25 µl of sterile water and dissolved for one hour at 56°C. The fresh DNA samples were immediately subjected to PCR experiments. The remainder of the DNA was aliquoted and stored at -20°C.

DNA extraction from cultures:

The procedure routinely performed at the Kuvin Center has been described by Eisenberger and Jaffe, (1999). The reference strains used as positive controls had been extracted by this method as well as every newly collected strain which was cultivated during this research. Parasites were harvested at a density of about 2 x 10^7 parasites/ ml. The cells were centrifuged for 10 minutes at 2500 rpm and washed three times with phosphate bufered saline (PBS). Proteinase K digestion and phenol-chloroform extraction followed. The protocol differed in some points from the extraction described above: Triton-X 100 (1%) was used instead of SDS. The proteinase K digestion was performed for 2h. The DNA was resuspended in 100 µl of TE buffer (1 mM EDTA; 10 mM Tris-HCl; pH 8.0). The DNA concentrations were determined photometrically at an absorbtion of 260 nm and stored at 4°C. Dilutions of 100 ng/µl and 20 ng/µl were prepared.

Guanidine extraction:

The method has been described by Boom *et al.*, (1990).

Preparation of silica beads:

Six g of silica beads (Sigma) were washed in 50 ml of sterile ddH_2O and mixed gently for 24 h at room temperature in a rotating hybridization oven. After a quick centrifugation the water was pipetted off. The silica beads were washed once again, the second time rotating for 5 h was sufficient. Sixty µl of HCl (1M) were added to the pellet. The consistency of the silica beads was viscous. The purified silica beads were transferred to a brown glass tube, autoclaved and stored at room temperature. The prepared silica beads were stable for long term use.

Extraction:

The tissue samples were transferred to 1.5 ml tubes containing 500 µl of guanidinium thiocyanate solution (4M guanidinium thiocyanate (GTC), 0.1 M Tris-HCl pH 6.4; 0.02 M EDTA, pH 8; 1.3% Triton X-100) and incubated over night at 56°C under gentle agitation. One ml of 6 M sodium iodate (NaI) and 10 µl of resuspended silica beads were added. The samples were vortexed and kept on ice for one hour. After a quick centrifugation (3 seconds at 12,000 rpm), the supernatant was carefully removed and discarded. Wash buffer (0.01M Tris-HCl, pH 7.5; 0.05 M NaCl; 1 mM EDTA pH 8; Ethanol 50%) was added to each tube. The silica bead

pellets were vortexed until they were in suspension. Another quick centrifugation followed and the buffer was removed. The pellets were washed with 100% ethanol and vacuum dried. They were resuspended in 100 µl of sterile ddH_2O and incubated for one hour at 56°C. The silica beads were spun down prior to PCR to avoid any inhibitory activity. The guanidine solution and the sodium iodate were freshly prepared for every new extraction. The wash buffer was stored at -20°C.

Chelex extraction:

A 5% Chelex solution was prepared by washing 500 mg of Chelex -100 (Biorad) in 10 ml of sterile ddH_2O. The suspension was boiled for 10 minutes in a water bath. After centrifugation for 5 minutes at 3000 rpm the water was removed. The washing step was repeated once. The purified chelex was resuspended in 10 ml of ddH_2O and aliquoted to 250 µl volumes for subsequent use. The samples were incubated over night at 56° and then heated for 10 minutes to 94 °C. After centrifugation of 1 minute at 12000 rpm the supernatant was directly used for PCR (Harris *et al.,* 1998).

Crude methods:

Lysis of cultured parasites:

PCR diagnosis from cultured strains was performed whenever new strains were cultivated in the laboratory and a differentiation between *L.major* and *L.tropica* was required. The density of parasites was not defined, since cultures were usually subjected to PCR as soon as there was a microscopical evidence of promastigotes. Cultures (100 µl) were transferred to 1.5 ml tubes and diluted 1:4 (300 µl) with ddH_2O. The cultures were boiled for 5 minutes to increase cell lysis. One µl of the lysate was used as template.

Crude preparation of dermal scrapings on filter paper:

Dermal scrapings were processed as for the phenol-chloroform extraction. After the over night digestion with proteinase K the samples were only heated for 10 minutes to 95°C (or boiled for 5 minutes) to inactivate the enzyme and then centrifuged shortly at high speed (12,000 rpm). The supernatant was directly used for PCR.

2.2.4.1. Choice of extraction methods according to specimens:

The patient samples (dermal scrapings on filter paper) were tested with each of the presented extraction methods. Some of the other sample groups were extracted with one specific method: The phenol-chloroform method was used for the jackal samples and the hyrax ear. A small part

(~0.5 g) of each jackal ear was chopped with a surgical blade and transferred to a 1.5 ml tube. Small pieces (~0.1 g) of the formalin fixed jackal spleens were cut out and were washed three times with sterile PBS prior to the extraction. Thorough purification was also required for Giemsa stained smears and paraffin embedded skin biopsies (phenol-chloroform). The guanidine extraction method was used for several series of patients samples (dermal scrapings on filter paper) and also for the parched hyrax tissues. The chelex extraction method was employed on dermal scrapings from patients and on the rodent samples. The crude extraction methods were used experimentally on filter paper samples from patients.

2.2.4.2. Extraction from paraffin embedded biopsies:

Four paraffin embedded skin biopsies of CL patients were proceeded. For deparaffinization, several slices were cut from each block and transferred into a 1.5 ml tube, containing 1 ml of xylene. The sample was vortexed and then left for 10 minutes at room temperature. After centrifugation for 5 minutes at 12,000 rpm the xylene was removed and the process repeated. One ml of absolute ethanol was added, the samples were mixed and centrifuged for 5 minutes and the ethanol was removed. The ethanol washing was repeated twice. Finally, the samples were vacuum dried, redisolved in ddH_2O and submitted to phenol-chloroform extraction (Laskay *et al*., 1995).

The 3 extraction methods (phenol-chloroform, guanidine and Chelex) were compared on infected mouse liver tissue, which had been evenly distributed on filter paper. Squares of 0.5 cm^2 were cut off the filter paper (6 pieces for each method, always 2 stored at a different temperature, see also the following passage).

2.2.4.3. Storage conditions:

The positive extraction control (mouse liver infected with *L.donovani*) was distributed homogenously on 3 pieces of filter paper. Each filter paper was stored at a different temperature (-20°C, 4°C and 20°C), in order to test the stability of the DNA in the tissue over a period of 1 year under different conditions. Squares of the same size (0.5 cm square) were cut from each filter paper and extracted in doublets by phenol-chloroform-, guanidine and chelex extraction.

2.2.5. PCR:

2.2.5.1. Oligonucleotides:

In the literature no primers were found which could achieve sensitive and species specific diagnosis for all species simultaneously. In order to adapt a diagnostic system to the specific

needs of the region, 3 different pairs of oligonucleotides were employed, which targeted at the kinetoplast minicircles (Uni21/Lmj4; 13A/13B and MP3H/ MP1L). The kinetoplast minicircles were selected as a suitable target due to the high copy number (10.000), implying the potential for high sensitivity. Close to the end of the study another pair of primers (LITSRn/L5.8S) was published (El Tai *et al.,* 2000) and introduced also to this work. These primers target the intergenic transcribed spacer (ITS) and fulfill all the requirements (sensitive and species-specific diagnosis).

1. Whole minicircle amplification with primers Uni21/Lmj4:

The primer pair (Uni21/Lmj4) had been specifically designed for the purpose of direct diagnosis and the differentiation of *L.major* and *L.tropica* in Israel, which was had been the initial aim of this study. This primer pair had not been tested previously and was suggested by Prof. Greenblatt, Kuvin Center. The primer pair Uni 21 (5' -GGGGTT GGTGTAAAATAGGCC - 3') and Lmj4 (5' - CTAGTTTCCCGCCTCCGAG - 3') was designed by Eresh, Axelrod, Greenblatt and Barker (Eresh *et al.,* 1993). The primer design was based on a published sequence from a *L.major* minicircle (Smith *et. al*., 1989). Primer Uni 21 was selected from a sequence within the conserved region. Primer Lmj4 was designed on the basis of the variable region of the same minicircle sequence.

2. Genus specific kinetoplast primers 13A/13B:

For the screening of desert rodents another PCR was employed, which needed to be primarily sensitive but not necessarily species specific. For this purpose kDNA primers 13A/13B (Rodgers *et al*., 1990) appeared to be suitable. Primers 13A (5'-GTGGGGGAGGGGCGTTCT-3') and 13B (5'-ATTTTCCACCAACCCC CAGTT-3') amplified a sequence of 120 bp of the kDNA minicircles. This sequence is conserved in all minicircle classes of all *Leishmania* species. Due to the small size of the sequence and its presence in all minicircles the PCR with primers 13A/13B is highly sensitive and therefore suitable for screening.

The following Figures demonstrate how primers Uni21/Lmj4 and primers 13A/13B relate to the minicircle, as a sequence in Figure 6 and as a circle in Figure 7.

Figure 6: Section of the *L.major* minicircle

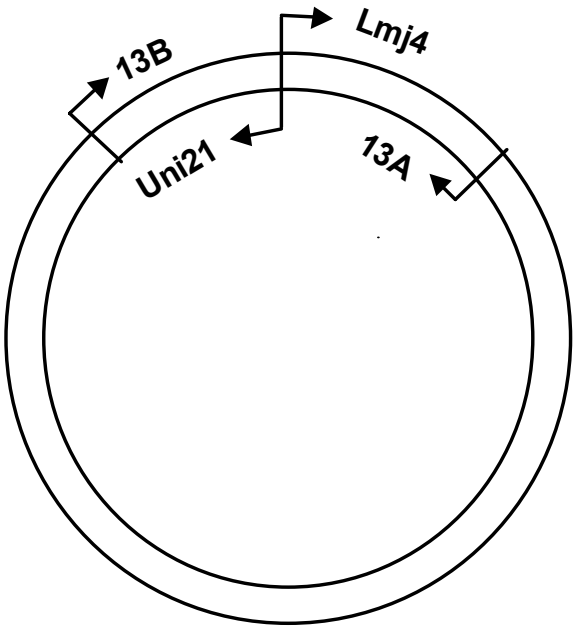
```
                                                                      50
L.major-original  AATTCCCCGA CCCACCCGGC CTATTTTACA CCAACCCCTA GTTTCCCGCC
           Uni21  .......... .← 3'-CCG GATAAAATGT GGTTGGGG-5'..........
            Lmj4  .......... .......... .......... ....5'-CTA GTTTCCCGCC
             13A  .......... .......... .......... .......... ..........
              13B .......... .......... 5'-ATTTTCCA CCAACCCCCA GTT-3'→...

                    51                                                100
L.major-original  TCCGAGCCCA AAAATGGCAA TTTTCGGCCA AAAATCGAAC GGGGTTTCTG
           Uni21  .......... .......... .......... .......... ..........
            Lmj4  TCCGAG-3'→.......... .......... .......... ..........
             13A  .......... .......... .......... .......... ..........
             13B  .......... .......... .......... .......... ..........

                    101                                               150
L.major-original  CACCCATTTT TCGAATTTCG CAGAACGCCC CTACCCACGG GACCAGAAAA
           Uni21  .......... .......... .......... .......... ..........
            Lmj4  .......... .......... .......... .......... ..........
             13A  .......... .....← 3'- TCTTGCGGG GAGGGGGTG -5'.........
             13B  .......... .......... .......... .......... ..........

                  151                                                 200
L.major-original  GTTTGAAATT TCGGGCATTT TTTGACCCCC ATAACCAAAA TACTCACATA
           Uni21  .......... .......... .......... .......... ..........
            Lmj4  .......... .......... .......... .......... ..........
             13A  .......... .......... .......... .......... ..........
             13B  .......... .......... .......... .......... ..........
```

Figure 7: kDNA minicircle with primers Uni21/Lmj4 and 13A/13B



Uni21/ Lmj4 → 650 - 850 bp

13A/ 13B → 120 bp

***L.braziliensis* specific kinetoplast primers MP3H/ MP1L:**

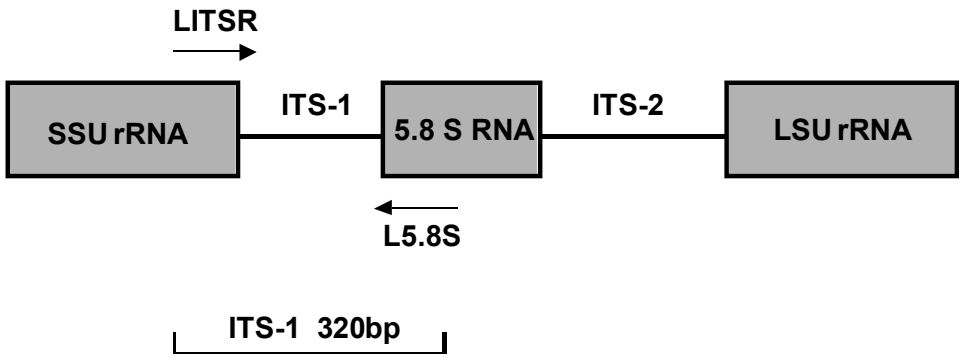
Primers MP3H (5'-GAACGGGGTTTCTGTATGC-3') and MP1L (5'- TACTCCCCGACATG

CCTCTG-3') were developed by Lopez *et al*., (1993) in order to distinguish between the species of the *L.braziliensis* and the *L.mexicana* complex. A 70 bp sequence specific of *L.braziliensis* minicircles is amplified. The *L.mexicana* complex as well as all Old World *Leishmania* species are not amplified.

Amplification of the ITS-1 region with primers LITSRn/L5.8S:

This non-kinetoplast based PCR has been recently published by El Tai *et al*., (2000). Primers LITSRn (5'- CTGGATCATTTTCCGATG - 3') and L5.8S (5'- AAGTGCGATAAGTGGTA-3') amplify a section of the intergenic transcribed region (ITS-1, about 300 bp) of all *Leishmania* species. In combination with the enzyme digestion of the PCR product using the restriction enzyme *Hae III* (= *BsuRI*) species specific diagnosis is achieved. ITS sequences of *L.d.infantum, L.major, L.tropica, L.braziliensis, L.guyanensis, L.panamensis, L.mexicana, L.amazonensis* and of other *Leishmania* species are published in the EMBL data base. These sequences are species specific and restriction sites could be identified that would allow the differentiation of all important *Leishmania* species, in the case of *L.donovani* and *L.braziliensis* the identification of the complex. (G. Schönian, personal communication). Figure 8 shows the ITS region and how primers LITSRn/L5.8S relate to it.

Figure 8: Internal transcribed spacer of the ribosomal operon



Restriction of the ITS-1 PCR-product:

The ITS-1 PCR products were digested with *BsuRI (HaeIII)* (MBI Fermentas, Lithuania). The amplified PCR products (8 µl) were mixed with 1 µl of enzyme and 1 µl of 10x buffer (10 mM Tris-HCl, pH 8.5; 10 mM $MgCl_2$; 100 mM KCl; 0.1 mg/ml BSA) in 0.5 ml tubes, and were incubated for 2 hours at 37°C.

2.2.5.2. PCR-conditions:

The regular volume of each PCR reaction was 25 µl. Only the ITS-1 amplifications were either performed in 25 or in 50 µl. The reaction mixtures were prepared on ice. All components were first pipetted into a 1.5 ml tube, well mixed and then aliquoted to 200 µl PCR tubes. For positive controls, 1 µl (20 ng/µl) of purified leishmanial DNA was used. When resuspended DNA from filter paper extractions was examined, 1 to 5 µl of extracted DNA were added. In case of lysed cultures 1 µl was sufficient as template. The reaction mix without DNA served as negative control.

Several different polymerases were employed. The Promega Taq polymerase (Promega, Madison, WI) was used as a standard enzyme for most of the reactions. It was generally employed for the PCR with primers 13A/13B and MP3H/MP1L and for initial studies with primers Uni21/Lmj4 on purified DNA. For the studies on dermal scrapings with primers Uni21/Lmj4 a polymerase with a higher activity and stability was employed in order to increase the chances for positive results. In the beginning the TaKaRa Ex Taq polymerase (Takara Shuzo co., LTD., Japan) was used. Later the Fermentas Taq polymerase (MBI Fermentas, Lithuania) proved to be more reliable and was adopted in the laboratory.

The reaction mix using the Promega Taq polymerase was composed of 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each nucleotide (dATP, dGTP, dCTP, dTTP), 1µM of each primer, 1 U of polymerase and up to 5 µl of template DNA. Primers MP3H/ MP1L required only 0.625 U of the polymerase, since the amplified sequence was smaller (70 bp). PCR with the TaKaRa Ex Taq polymerase was performed in 10x Ex Taq buffer, 2 mM $MgCl_2$, 200 µM of each nucleotide, 1 µM of each primer, 0.5 U of TaKaRa Ex Taq polymerase and up to 5 µl of template DNA. The PCR with the Fermentas enzyme was performed in 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 200 µM of each nucleotide, 1 µM of each primer, 1 U of Fermentas Taq polymerase and 1-5 µl of template DNA. Table 3 gives details on the 4 different PCR methods used in this study.

Table 3: PCR methods

1. PCR with primers Uni21/Lmj4:

reagent	original conc.	µl	final conc.
ddH_2O		15.0-19.0	
buffer	10 x	2.5	1 x
Mg^{2+}	25 mM	1.5	1.5 mM
Primer Uni21	100 µMol	0.25	25 pmol/25 µl
Primer Lmj4	100 µMol	0.25	25 pmol/25 µl
dNTP's	20 mM	0.25	0.2 mM
Taq-Polymerase	5 U/µl	0.25	0.625 U/ 25µl
DNA		1-5	
final volume		25	

2. PCR with primers 13A/13B:

reagent	original conc.	µl	final conc.
ddH_2O		13.5-17.5	
buffer	10 x	2.5	1 x
Mg^{2+}	25 mM	1.5	1.5 mM
Primer 13A	100 µMol	1.0	100 pmol/25µl
Primer 13B	100 µMol	1.0	100 pmol/25µl
dNTP's	20 mM	0.25	0.2 mM
Taq-Polymerase	5 U/µl	0.2	1U/ 25µl
DNA		1-5	
final volume		25	

3. PCR with primers MP3H/MP1L:

reagent	original conc.	µl	final conc.
ddH_2O		13.5-17.5	
buffer	10 x	2.5	1 x
Mg^{2+}	25 mM	1.5	1.5 mM
Primer MP3H	100 µMol	1.0	100pmol/25µl
Primer MP1L	100 µMol	1.0	100pmol/25µl
dNTP's	20 mM	0.25	0.2 mM
Taq-Polymerase	5 U/µl	0.125	0.625 U/µl
DNA		1-5	
final volume		25	

4. PCR ITS with primers LITSRn/L5.8S

reagent	original conc.	µl	final conc.

ddH_2O		33.0-37.0	
buffer (Promega)	10 x	2.5	1 x
Mg^{2+}	25 mM	3	1.5.mM
Primer LITSRn	100 µMol	1.0	25 pmol/50µl
Primer L5.8S	100 µMol	1.0	25 pmol/50µl
dNTP's	20 mM	4.0	0.2 mM
Taq-Polymerase	5 U/µl	0.4	2U/50µl
DNA		1-5	
final volume		50	

Conc.= concentration. The volume of the water has to be adapted to the volume of the template used: more template DNA has to be balanced by using less water. The same has to be considered if additives are used. The final reaction volume has to be regarded. Other volumes can be used, if concentrations stay the same. Eg.: PCR 4 was also used as a 25 µl mix, using exactly the half amount of every substance.

Cycling was performed in a minicycler (M.J. Research, Watertown, USA). Mineral oil was not required because the machine was equipped with a heating device from above, in order to prevent evaporation within the PCR tubes. Table 4 shows the different cycling protocols for each PCR-method (as finally established).

2.2.6 Agarose gels:

1.5% or 2.5% agarose gels were prepared by dissolving 1.5 g respectively 2.5 g of agarose (Seakem LE agarose, FMC Bioproducts, Rockland, Maine, USA) in 100 ml of TAE buffer (0.04 M Trisacetate; 0.0001 M EDTA) in an Erlenmeyer flask. The percentage of agarose depended on the size of the PCR product. For the amplificates with primers Uni21/Lmj4 (680-850 bp) a 1.5% agarose gel and for the amplificates with primers 13A/13B (120 bp) and MP3H/MP1L (70 bp) a 2.5% gel was prepared. The agarose was dissolved by boiling in a microwave oven for 2-4 minutes, until the solution was transparent. After cooling the agarose to about 60-70°C ethidium bromide (2.5 µl) was added. The well mixed fluid was poured into a gel-cast (DNA plus, USA Scientific) and was left to solidify.

For higher resolution, 5 µl of Gel star stain (FMC BioProducts, Rockland, Maine) was used instead of ethidium bromid. Gel star stain was employed for the analysis of the restriction patterns obtained after digestion of the ITS-1 PCR-products (3% agarose gel) as well as for the amplification of filter paper samples with primers Uni21/Lmj4 (the least sensitive primers).

Table 4: Cycling

1. PCR with primers Uni21/Lmj4:

Preheating	5 min	94°C	
Denaturation	1 min	94°C	
Annealing	80 sec	60°C	35-45 cycles
Elongation	1 min	72°C	
Extension	10 min	72°C	

2. PCR with primers 13A/13B:

Preheating	3 min	94°C	
Denaturation	1 min	94°C	
Annealing	1 min	50°C	30 cycles
Elongation	1 min	72°C	
Extension	10 min	72°C	

3. PCR with primers MP3H/MP1L:

Preheating	3 min	94°C	
Denaturation	1 min	94°C	
Annealing	1 min	54°C	35 cycles
Elongation	1 min	72°C	
Extension	10 min	72°C	

4. PCR of the ITS-1 region with primers LITSRn and L5.8S:

Preheating	4 min	95°C	
Denaturation	40 sec	95°C	
Annealing	30 sec	53°C	36 cycles
Elongation	1 min	72°C	
Extension	6 min	72°C	

Ten µl of each PCR product were loaded with 2.5 µl of 5x bromophenol blue loading buffer (BPB) on the gel. A few µl (2-3µl) of molecular size markers PGEM, ϕHAE or ϕHinf (100 ng/µl) were loaded as controls. PGEM was used for the larger PCR-products, ϕHAE or ϕHinf were used for the small amplificates. Electrophoretic separation was performed at 120 V and 120 mA for about 40 minutes. The PCR products were visualized under UV-light and photographed (ImageMaster VDS, Biotech Pharmacia). The pictures were captured with the NIH-Image program.

2.2.7. Avoiding inhibition:

Several experiments were performed using additives in the PCR, in order to reduce possible inhibition caused by substances, which were not sufficiently eliminated by the crude extraction (such as hemoglobin). Bovine serum albumin (BSA) or dimethyl-sulfoxyd (DMSO) in combination with formamide were used. BSA was sterile filtrated and used in a concentration of 10 mg/ml. The effect of BSA was examined by adding 0, 1, 2 and 4 µl to 4 reactions performed with 1 µl of crude template.

A similar experiment was performed using 2.5 % DMSO and 1% formamid in three reactions, containing 1, 2.5 and 5 µl of crude template. As a comparison, 3 reactions without additives, with 1, 2.5 and 5 µl of crude template were prepared and amplified.

2.2.8. Avoiding contamination:

The potential sensitivity of the PCR was theoretically up to less than one parasite. This implied a high risk for contamination, which had to be strictly avoided. As a general rule, the extraction of DNA, the preparation of the PCR and electrophoresis of the PCR products should be strictly separated. It is also recommended to use different sets of pipettes for the different parts of the work. Due to limitations of space and technical equipment this standard could not be met. Instead, extra precautions had to be taken:

- DNA extraction from clinical material and preparation of the PCR was performed in a hood, located in a *Leishmania*-free room. The parts of the work, which were less sensitive and which were by themselves a potential source of contamination, were performed in a separate room (PCR-cycling, gel electrophoresis and also the extraction from cultured parasites)
- Gloves were changed during the procedures from time to time.
- The working surface in the hood was bleached with 5% bleach and UV-radiated prior to extraction or PCR.

- Before every new extraction or PCR the pipettes were wiped with 5% bleach and UV-radiated from both sides for at least 15 min. This was necessary, because only one set of pipettes was available, which was used alternately for highly sensitive purposes and for very concentrated DNA (PCR- amplified or extracted DNA from cultures).
- The micro-centrifuge was bleached prior to extractions since the tops of the 1.5 ml tubes came in contact with the upper margin of the cups holding the tubes. Since normally highly concentrated DNA was centrifuged in the same centrifuge these cups were assumed to be a source of contamination.
- A security distance between the 1.5 ml tubes was kept by placing the tubes in distances to each other. By this, the tops of neighbouring tubes did not touch each other when opened.
- Filter tips were used for extraction and PCR.
- Whenever possible 20 µl tips were used instead of 10 µl tips. Due to the length of the 20 µl tips the shaft of the pipette could not touch the 1.5 ml tubes from inside. For volumes below 4 µl the 10 µl pipette was used to ensure accuracy.
- For extraction tight tubes had to be used since they were to rotate in a hybridization oven. Only screw-cap 1.5 ml tubes proved to be tight enough for this purpose.

2.2.9. Controls:

1. Negative controls:

The extraction as well as the PCR had to be monitored strictly for possible contamination. Two negative extraction controls were routinely used for every extraction series. A piece of sterile filter paper and a piece of filter paper with a drop of human blood (without history of previous infection with leishmaniasis) were extracted parallel to the samples. In order to exclude non-specific results in the rodent samples, ear tissue from a negative *Psammomys* was used as negative control. The tissue was collected from a laboratory animal of the Diabetes Unit of the Hadassah Hospital, Ein Karem (received from Dr. Ehud Ziv). For the other animal species no suitable negative controls were available. The PCR itself was monitored by one reaction in every PCR, which contained only the reagents and no template.

2. Positive controls:

Liver tissue of a laboratory mouse, which had been infected with *L.donovani*, was blotted on filter paper. A piece of about 0.5 cm in diameter was used as a positive control for the extraction. As an amplification control and also as a reference, 20 ng of DNA from at least one of the *Leishmania* reference strains was amplified in every experiment.

2.2.10. Solutions:

Agar	2.5 g agar-agar 1 g peptone 0.5 g NaCl 0.3 g Beef extract (Difco) 100 ml ddH_2O
Agarose gel (1.5-3%)	1.5/ 2.5/ 3 g agarose 100 ml of 1x TAE buffer
Bromo-phenol-blue (1x BPB)	0.04% BPB 0.04% xylene cyanol 5% glycerol in H_20
Chelex (5%)	500 mg chelex-100 filled to 10 ml with ddH_2O
Chloroform	24 vol chloroform 1 vol isoamylalcohol
EDTA (0.5M, pH 8.0)	186.1 g of disodium ethylendiaminetetraacetate $2H_2O$ add to 800 ml with ddH_2O pH-adjustment with NaOH pellets
Ethidium bromide	10 mg/ml in ddH_2O
Guanidine solution	4 M GTC (mw 118.2), 47.28 g 0.1 M Tris-HCl (pH7.5), 10 ml (of a 1 M solution) 0.02 M EDTA (pH8.0), 4 ml (of a 0.5 M solution) 1.3% Triton x-100, 1.3 ml filled up to 50 ml
Locke's solution	9.2 g NaCl 0.24 g $CaCl_2$ 0.15 g $NaHCO_3$ 0.42 g KCl 1 g dextrose 1000 ml ddH_2O
NNN medium Solid phase: liquid phase:	 Agar 10% defibrinated rabbit blood Schneider's medium overlaid on the solid phase
NET-buffer	50 mM NaCl 10 mM EDTA 50 mM Tris (pH 7.4) ddH_2O made up to 100 ml
Phosphat buffer for	0.7 g KH_2PO_4

Giemsa straining	1.0 g Na_2HPO_4 add ddH_20 to 1 liter
Phosphat-buffered saline (PBS)	8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH_2PO_4 adjust pH to 7.5 with HCl add ddH_20 to 1 l
Schneider's stock	1 package powered Schneider's 4 l ddH_2O 10 g yeastolate 134 mg L-cystine: diHCl 3.6 g L-tyrosine: Na(disodium) 250 ml 10 N NaOH pH-adjustment to pH 6.9 with 1.0 N HCl (about 175 ml) adjust volume to 5 liter
Schneider's drosophila medium	170 ml Schneider's stock solution 30 ml fetal calf serum 4 ml L-glutamin 200 U/ml penicillin (20 µl of 200,000 U/ml penicillin) 200 mg/ml streptomycin (20 µl of 200mg/ml)
Semi-solid medium (SS)	7 parts of Locke's solution 1 part of melted agar 10% defibrinated rabbit blood Antibiotics (200 U/ml penicillin, 200 mg/ml streptomycin)
Silica beads	3 g silica beads 30 µl of 1M HCl resuspended in 1 ml ddH_2O (protocol of preparation in text)
Sodium acetate (NaAc) 3M, pH 6	408.1 g NaAc $3H_20$ for pH-adjustment glacial acetic acid ddH_20 filled up to 1 l
Sodium iodate (NaI), 6 M	45 g NaI (mw 150) ddH2O made up to 50 ml
TAE (50x)	242 g tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)
TAE (1x)	20 ml of 50x TAE Made up to 1 liter with ddH_2O
TE (pH 7.4)	10 mM Tris HCl (pH 7.4) 1 mM EDTA (pH 8.0)

TE (pH 8)	10 mM Tris HCl (pH 8.0) 1 mM EDTA (pH 8.0)	
Tris (1M)	121 g tris base in 800 ml of ddH_2O pH-adjustment with HCl	
Washing buffer (20 x) 2. M NaCl	0,2 M Tris-HCl (pH7.5) 20 ml (of 5 M NaCl) 20 mM EDTA (pH 8.0) ddH_2O made up to	20 ml (of 1 M Tris-HCl) 4 ml (of 0.5 M EDTA) 100 ml
Washing buffer (1x)	25 ml of 20x washing buffer 225 ml ddH_2O 250 ml ethanol (100%)	

2.2.11. List of reagents:

Agarose (LE agarose)	Seakem, Rockland Maine, USA
BsuRI (HaeIII)	MBI Fermentas, Lithuania
Chelex 100 Resin	Bio-Rad
dNTP	MBI Fermentas, Lithuania
Gelstar nucleic acid stain	FMC BioProducts, Rockland, Maine
Giemsa's staining solution	Gurr, BDH, England
Guanidine-thiocyanate	Fluka
L-cystine dihydrochloride	Sigma
L-tyrosine disodium	Sigma
Molecular size markers (PGEM, ϕHAE, ϕHinf)	Promega
Oligonucleotides	Genset SA, France
Proteinase K	Sigma
RNase A	Sigma
Schneider's Drosophila medium powder	Gibco
Silica beads	Sigma
Sodium iodate	Merck
Taq polymerase	MBI Fermentas, Lithuania, Germany
Taq polymerase	Promega, Madison, Wi
Taq polymerase (TaKaRa Ex Taq)	TaKaRa Biomedicals, Shuzo CO., LTD, Japan
Triton X-100	BDA
Yeastolate (bacto-yeast-extract)	Difco

3. Results

Several different extraction- and PCR-methods have been employed in this study. Methods have been changed and were even substituted as soon as better methods were available. Different sample groups have been studied, which were collected (or received) at different times during the study period, depending mainly on external circumstances. Some sample goups (eg. *Psammomys,* desert rodents) have been proceeded with the purpose of evaluating the quality of the method. As a "byproduct", interesting and sometimes unexpected results were found, as for example a new host species. Therefore technological findings or improvements are inseparably connected with the specific results obtained from different sample groups. This chapter is mainly structured according to the methods used.

3.1. Efficiency of the tested DNA extraction methods:

A major objective has been to find the most efficient and practicable extraction method with particular regard to the dermal scrapings on filter paper. It was important to find a reliable method which could be adopted to a clinical laboratory for routine diagnosis. The results with the different extraction methods are summarized here and will be thoroughly discussed in chapter 4 (Discussion).

All tested extraction methods were successful. The classical phenol-chloroform method was applied on several series of dermal scrapings of patients (mostly the Hadassah patients at the beginning of the study). Differently preserved samples were occasionally received too (eg. Giemsa stained smears, paraffin embedded skin biopsies, formalin fixed jackal spleens). These samples were proceeded with the phenol-chloroform extraction, because this method had the greatest potential to remove inhibitory agents. The tested Giemsa stained smears (positive by microscopy) were PCR-positive (3 out of 3), as well as the paraffin embedded biopsies (4 out of 4). The formalin fixed tissue was not amplified with *Leishmania* specific primers, but yielded a weak signal with mammalian primers (performed by Carney Mattheson). This proved that formalin fixed tissue can be theoretically proceeded, but that inhibition has to be expected. The phenol-cloroform extraction is labor-intensive and difficult to handle in a non-research oriented laboratory. Due to the frequent re-opening of the 1.5 ml tubes, the risk of sample cross-contamination was highest with this method.

For these reasons other extraction methods were tested too: At first, experiments with crude extractions were performed, using only the first steps of the phenol-chloroform extraction (lysis and digestion, concluded by a short boiling step). Experiments with this crude method were

performed on dermal scrapings from CL lesions in the initial phase of the study, when only the kDNA primers Uni21/Lmj4 were employed. The success rate was sometimes high, (up to 4 positives out of 5 tested samples), and sometimes the results were poor (none, 1 or 2 positives in a series of 5-10 samples). Conditions were changed each time in order to finally introduce the best methodology (eg. amount of template, additives in the PCR, cycling temperatures). It was obvious that inhibition was the main cause for sub-optimal results: small amounts of template (1-2 µl lysate) had a far better success rate than larger amounts (5 µl), the addition of BSA reduced the inhibition by hemoglobin.

The guanidine extraction was tested as another potential alternative to the phenol-chloroform extraction. It also produced good results, on patient samples (mainly Tel Hashomer Hosital, Wadi Albethan) and on the dog peripheral blood spots (on filter paper). The guanidine method was easier to handle and also quicker than the phenol-chloroform extraction.

The Chelex extraction was only "discovered" much later as another possible extraction method. It has been employed on the rodent samples (ear scrapings from *Psammomys* and *Gerbilli*). Since 28 out of 30 *Psammomys* samples (ear scrapings on filter paper) were strongly positive it can be stated that this method is highly efficient. These samples were characterized by a high tissue and a low blood content. The Chelex method was also employed on individual patients, but it has not been evaluated yet on a larger number of patient samples. One patient sample (dermal scraping) was incubated for 1 hour in Chelex (not overnight) and was amplified without problems. An experiment with one sample (infected mouse liver, spotted on filter paper) which was incubated for either 1, 2, 3 or 4 hours showed that a longer incubation was indeed more efficient (more PCR product, stronger bands on the gel). The Chelex method was even more simple than the previous crude method (lysis, digestion, boiling) so that it could replace the latter.

No differences have been noticed between the 3 extraction methods (phenol-chloroform, guanidine and Chelex) when compared with practically identical samples (infected mouse liver spotted homogenously on filter paper, squares of 0.5.cm^2, 6 pieces for each method).

3.1.1 Suitability of different specimens for PCR diagnosis:

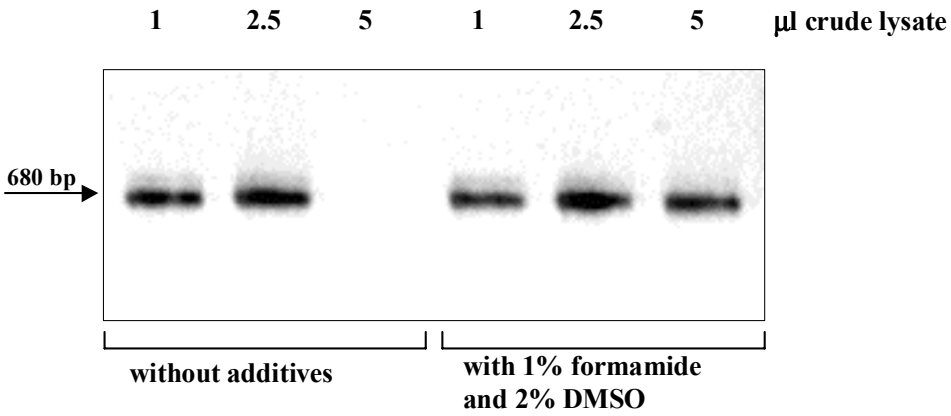
It was examined whether dermal scrapings (preserved on filter paper) would in fact be suitable for direct PCR diagnosis of CL. The sampling method and way of preservation proved to be efficient, as seen in several study groups (Hadassah patients, rodent ear scrapings, dog blood, Wadi Albethan inhabitants, Tel Hashomer patients). Filter paper samples (infected mouse liver spotted on filter paper) were stable at all three temperatures (20°C, 4°C, -20°C) for more than one year and were amplified equally well. Dermal scrapings on filter paper were examined

predominantly, but it was also relevant to test other types of samples. It was especially of interest to obtain results from early cultures in which promastigotes had been just detected. A crude preparation from Schneider´s medium (lysis in H_2O and boiling for 5´minutes) was amplified well (no inhibition seen). Inhibition was observed when blood containing media were used (eg. semisolid media). Peripheral blood spots on filter paper (from dogs with CVL), Giemsa stained smears and paraffin embedded skin biopsies proved to be suitable too.

3.1.2 Experiments with crude samples:

Inhibition was observed as a regular phenomenon when crudely extracted samples were subjected to PCR. The experiments with additives for the prevention of PCR-inhibition showed the following: bovine serum albumin (BSA) clearly helped to amplify crude samples containing hemoglobin. The crude template (1 µl) was amplified well in the presence of 4 µl BSA (10 mg/ml), less amplified with 2 µl of BSA, very faintly amplified with 1 µl BSA and not amplified without BSA (results not shown). The experiment with 2.5 % DMSO and 1% formamid also showed a positive effect: When the additives were used the reaction tolerated 1, 2.5 and 5 µl of template. Without the additives the reaction with 5 µl of crude template was inhibited. Results are shown in Figure 9.

Figure 9: Experiment with additives against inhibition:



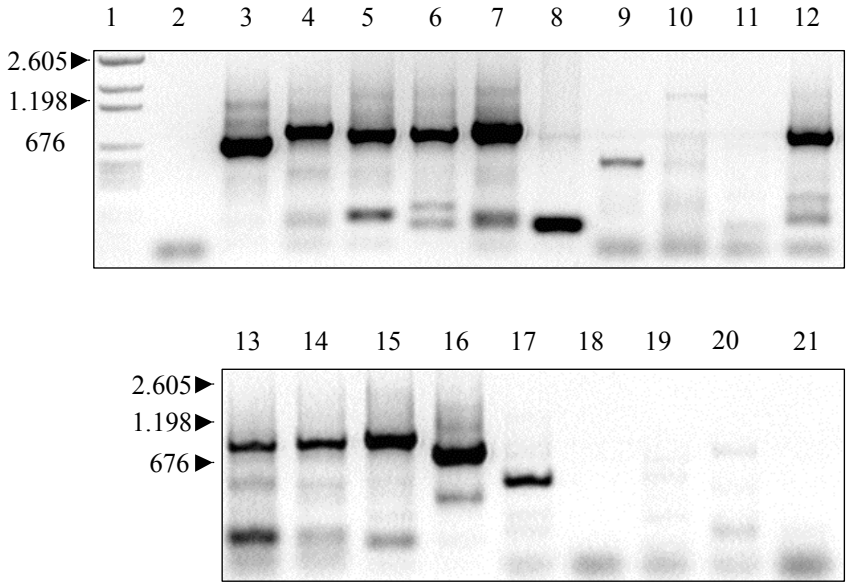
PCR with kDNA primers Uni21/Lmj4 with crudely extracted DNA from dermal scrapings on filter paper.

3.2. Amplification of whole minicircle DNA with primers Uni21/Lmj4:

3.2.1. Results with DNA purified from cultured reference strains:

It was found that the size of the PCR product was consistent within each species. Whole minicircles of kDNA were amplified, the PCR products therefore reflected the size of the minicircles in each species, ranging from 680 bp (*L.major*), 800 bp (*L.donovani* complex), 820 bp (*L.tropica*) to 850 bp (*L.aethiopica*). *L.major* could easily be differentiated from *L.tropica* and the *L.donovani* complex by the size of the PCR products, which had been one important aim of the study. *L.tropica* and the *L.donovani* complex could not be distinguished certainly since both produced similar sizes of bands. The minor bands were not always seen. The appearance of these bands depended on the PCR conditions (eg. the Taq-polymerase). Even though some of them seemed to be related to the species they were not consistent enough to be used for species specific identification. The New World species (*L.mexicana, L.braziliensis, L.guyanensis, L.panamensis, L.amazonensis and L.d.chagasi*) could be clearly distinguished from the Old World species either by not amplifying at all or yielding much smaller products. Only *L.d.chagasi,* as a species belonging to the *L.donovani* complex, was amplified (820 bp). Species of *Leishmania* that are not pathogenic to humans were amplified: *L.gerbilli, L.turanica* and *L.killicki* yielded a product of 800 bp. A 680 bp product was obtained from *L.arabica*. Results with reference strains are shown in Figure 10:

Figure 10: PCR with primers Uni21/Lmj4 on different *Kinetoplastidae* (20 ng of DNA)

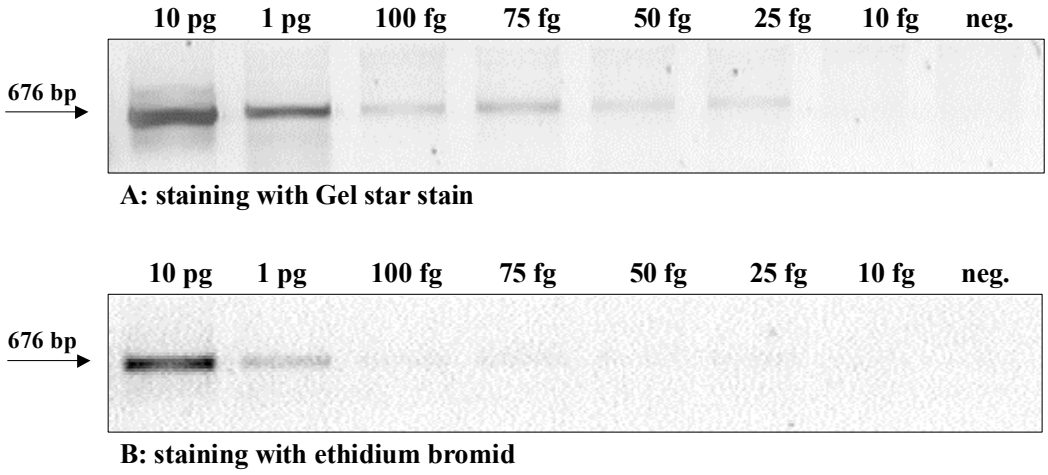


Lane 1 DNA size marker PGEM; lane 2 neg. PCR-control; lane 3 *L.major*; lane 4 *L.tropica*; lane 5 *L.donovani*; lane 6 *L.d.infantum*; lane 7 *L.aethiopica*; lane 8 *L.m.mexicana*; lane 9 *L.braziliensis*; lane 10 *L.b.guyanensis*; lane 11 *L.m.amazonensis*; lane 12 *L.d. chagasi*; lane 13 *L.gerbilli*; lane 14 *L.turanica*; lane 15 *L.killicki*; lane 16 *L.arabica*; lane 17 *Leptomonas Seymourii*; Lane 18 *Crithidia*; lane 19 *T.cruzi*; lane 20 *T.lousii*; lane 21 *P.davidii*.

Kinetoplastidae other than *Leishmania* were not amplified with primers Uni21/Lmj4 (*Trypanosoma cruzi, Trypanosoma lewisii, Crithidia fasciculata,* and *Phytomonas davidi).* The DNA of *Leptomonas seymouri* was amplified but the product was considerably smaller (500 bp). Primers Uni21/Lmj4 are specific for the Old World species of *Leishmania,* with the only exception of *L.d.chagasi* (identical to *L.d.infantum* in the Old World).

The sensitivity of the PCR assay with primers Uni21/Lmj4 was examined with a serial dilution of *L.major* DNA. If PCR products were visualized with Gel star staining, maximum sensitivity was found to be at 25 fg of target DNA (less than one parasite). With regular ethidium bromide staining, dilutions of less than 1 pg were almost undetectable. The sensitivity study is shown in Figure 11:

Figure 11: Comparison of sensitivity with Gel Star staining and ethidium bromide



PCR with kDNA primers Uni21/Lmj4, purified *L.major* DNA:

3.2.2. Results from lysed cultured isolates with primers Uni21/Lmj4:

PCR proved to be reliable whenever cultures of promastigotes cultivated in Schneider´s medium were used as lysates. If the cultures were bloody because of the medium (NNN and semisolid medium contain rabbit blood), the PCR reaction sometimes failed. Most patients had CL, acquired in Israel, where *L.major* and *L.tropica* were expected to be the causative agents. The smaller product (680 bp) was identified as *L.major,* whereas the larger product (about 800 bp) was either amplified from *L.tropica* or from the *L.donovani* complex. Based on the patient history (CL or VL, origin of infection) and on additional tests (eg. EF), the species was determined as being either *L.tropica* or of the *L.donovani* complex. Culture 14 (Table 5, LRC-

747) was derived from a sandfly trapped in a cave near Kfar Adumim (Photo 13) in the Judean Desert (trapped by Alon Warburg, Kuvin Center). It yielded a product of about 800 bp, which practically confirmed the suspected species of *L.tropica*. (The focus was already known to be endemic with *L.tropica*, since cutaneous infections by *L.d.infantum* have never been reported in the country). *L.tropica was* later confirmed by IFA, EF and PPIP-PCR (by colleagues at the Kuvin Center). Interestingly, *L.major* has also been identified from Kfar Adumim residents in 3 patients (Tables 5 and 6). *L.major* transmission in this area is possible too, since the Jordan Valley with a high *L.major* incidence is very close and endemicity in the settlement itself can not be excluded.

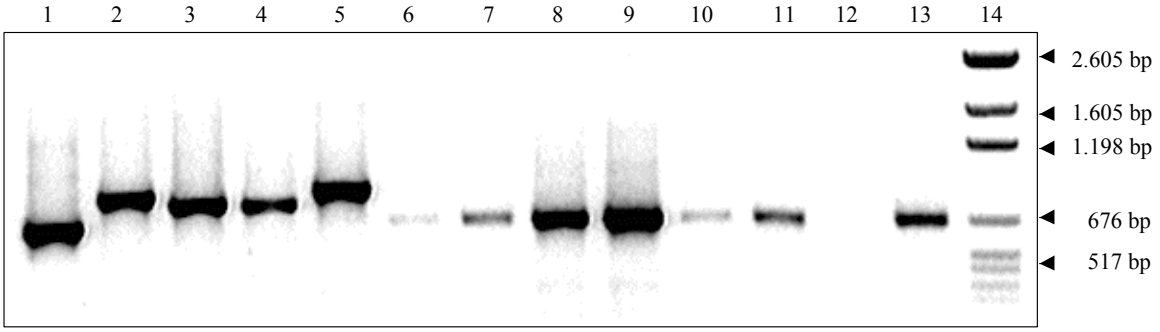
Culture 19 (LRC-744) was derived from a child with VL in Costa Rica. (The strain was brought to Israel by Lionel Schnur, Kuvin Center). It yielded a PCR product of 800 bp. *L.d.chagasi* was diagnosed since no other American *Leishmania* species would have been amplified. Table 5 and Figure 12 show the results from lysed cultures.

Table 5: PCR results with Uni21/Lmj4 from lysed cultures:

No.	*Sample*	**Smear**	**PCR**	**Diagnosis**	**Origin**
1	P1092	+	680 bp	*L.major*	Negev
2	P1111	++	680 bp	*L.major*	Kfar Adumim
3	P1120	+	680 bp	*L.major*	Jericho
4	P1130	+	680 bp	*L.major*	Jericho
5	P1132	-	680 bp	*L.major*	Jordan Valley
6	P1135	+	680 bp	*L.major*	Jericho
7	P1136	-	680 bp	*L.major*	Jericho
8	P1125	-	680 bp	*L.major*	Jordan Valley? Negev?
9	P1161	+	680 bp	*L.major*	Israel
10	P1165	-	680 bp	*L.major*	India?/Israel?
11	P2001	n.a.	680 bp	*L.major*	Israel
12	LRC-757	n.a.	820 bp	*L.tropica*	Kfar Adumim, sandfly
13	LRC-768	n.a.	820 bp	*L.tropica*	Israel
14	LRC-747	n.a.	820 bp	*L.tropica*	Kfar Adumim, sandfly
15	LRC-765	n.a.	680 bp	*L.major*	India
16	LRC-767	n.a.	680 bp	*L.major*	India
17	LRC-773	n.a.	680 bp	*L.major*	Israel
18	LRC-691	n.a.	680 bp	*L.major*	Israel
19	LRC-744	n.a.	800 bp	*L.d.chagasi*	Costa Rica
20	LRC-762	n.a.	680 bp	*L.major*	Uzbekistan

The LRC-number is given to newly collected strains of the *Leishmania* Reference Center, Jerusalem. The corresponding smears were not available (n.a.). P = patient of the Hadassah Hospital, Jerusalem.

Figure 12: PCR with primers Uni21/Lmj4 on lysed cultures:



Lane 1 *L.major*; lane 2 *L.tropica*; lane 3 *L.donovani*; lane 4 *L.d.infantum*; lane 5 *L.aethiopica*; lane 6 patient 1120 (Jericho); lane 7 patient 1125 (Negev); lane 8 patient 1130 (Jericho); lane 9 patient 1132 (Jordan Valley); lane 10 patient 1135 (Jericho); lane 11 patient 1136 (Jericho); lane 12 neg. PCR-control; lane 13 vaccination strain (Uzbekistan).

3.2.3. Results with primers Uni21/Lmj4 with dermal scrapings on filter paper:

This part of the work served to establish methods, which required experimental changes of conditions almost in every extraction series and PCR. This resulted in different success rates. Therefore the results of this part of the work are presented as a collection of positive results without a statistical evaluation.

Skin scrapings of patient lesions (spotted onto filter paper) proved to be useful for the diagnosis of CL, in patients as well as in reservoir animals. Positive results helped in species diagnosis, negative results could not exclude infection**.** It turned out that a low success rate was often due to inhibition. Series in which 2 µl of template were used had a better success rate than 5 µl eg.. The DNA lost its quality quickly (resuspension in H_2O), limiting the possibility of re-amplification of the relatively large PCR-product (680-850 bp). Out of this reason it was difficult to compare different PCR conditions with the same samples. The best possible DNA quality was necessary to obtain results, therefore freezing was not considered in this case (with smaller fragments no problem, see later). Out of filter paper samples from 100 suspected CL patients 24 were found positive with primers Uni21/Lmj4, either with the regular phenol-chloroform extraction or with the crude method (proteinase-K digestion, lysis and boiling). The first extractions yielded excellent results (also with the crude method!) as shown in Figures 13 and 14, whereas later extractions sometimes failed completely, with not even one single positive in 10 samples (mostly due to inhibition). The PCR from filter paper was usually successful when the corresponding smears contained a high number of amastigotes. When the smears (Giemsa stained) contained only single amastigotes the PCR with primers Uni21/Lmj4 (using the corresponding filter paper

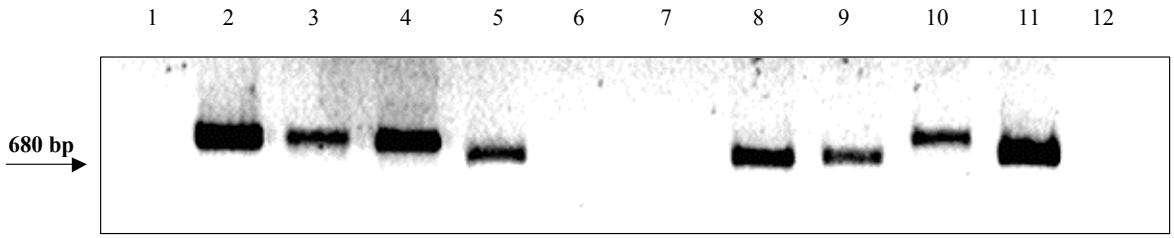
sample) the method was often not sensitive enough. Most patients were diagnosed with *L.major* (23 out of 24), one patient was diagnosed with *L.tropica* (Figure 13 lane 10, Table No. 6, patient 6). However, 6 patients with CL were diagnosed by PCR in whom microscopy and culturing had failed. Table No. 6 represents the results with whole amplified minicircles (Uni21/Lmj4) on dermal scrapings (preserved on filter paper).

Table 6: Results with primers Uni21/Lmj4 from skin scrapings preserved on filter paper:

No	Patient	Smear	Culture	PCR	Diagnosis	Origin of infection
1	1075	-	-	680 bp	*L.major*	Ethiopia/ Israel ?
2	1077	+	-	680 bp	*L.major*	Sinai, Arava
3	1078	-	-	680 bp	*L.major*	Israel
4	1083	-	-	680 bp	*L.major*	Dead Sea
5	1087	+	-	680 bp	*L.major*	Dead Sea
6	**1070**	**++**	**-**	**820 bp**	***L.tropica***	**Hemdat**
7	1088	++	-	680 bp	*L.major*	Negev
8	1095	+	-	680 bp	*L.major*	Jordan Valley
9	**1100**	**++**	**-**	**680 bp**	***L.major***	**Holon**
10	**1101**	**++**	**-**	**680 bp**	***L.major***	**Holon**
11	**999**	**++**	**-**	**680 bp**	***L.major***	**South America/Israel ?**
12	**1102**	**+**	**-**	**680 bp**	***L.major***	**South America/Israel ?**
13	1108	+	-	680 bp	*L.major*	Jordan Valley?
14	1124	-	-	680 bp	*L.major*	Kfar Adumim
15	1111	++	+	680 bp	*L.major*	Kfar Adumim
16	1113	+	+	680 bp	*L.major*	Qziot, Negev
17	1135	+	+	680 bp	*L.major*	Jericho
18	1150	++	-	680 bp	*L.major*	Jericho
19	1152	+	-	680 bp	*L.major*	Jordan Valley
20	1143	-	-	680 bp	*L.major*	Baghdad/Israel ?
21	1164	-	-	680 bp	*L.major*	Sinai
22	1181	+	-	680 bp	*L.major*	Qziot, Negev
23	1193	++	-	680 bp	*L.major*	Sinai
24	**LRC-762**	**?**	**+**	**680 bp**	***L.major***	**Uzbekistan**
25	**3 *Psammomys***	**+**	**n.a.**	**680 bp**	***L.major***	**Qziot, Negev**
26	**Mouse liver**	**+**	**+**	**800 bp**	***L.donovani***	**Infection in the laboratory**

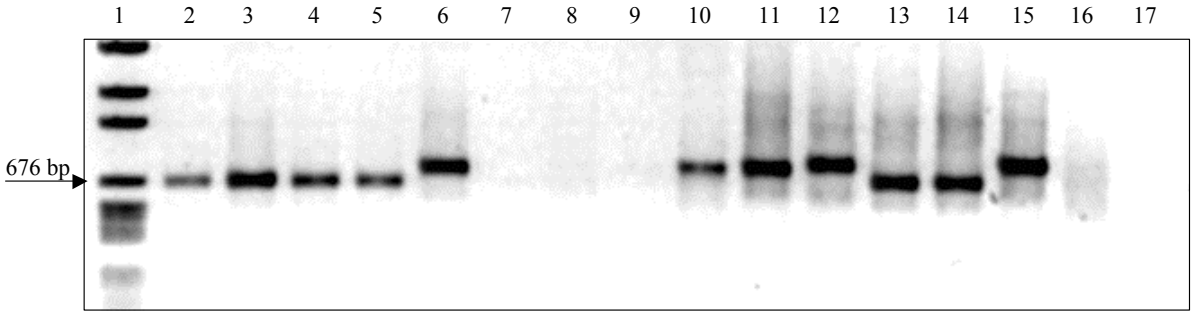
n. a.- not available. For better understanding: The origin of infection relates to the patient history. It contains the information obtained from the patient before the diagnosis was made.

Figure 13: PCR with primers Uni21/Lmj4 on crudely extracted filter paper samples:



Lane 1 *T.cruzi*; lane 2 *L.aethiopica*; lane 3 *L.donovani*; lane 4 *L.tropica*; lane 5 *L.major*; lane 6 negative PCR-control; lane 7 patient 1084 (IL); lane 8 patient 1086 (Qziot, IL); lane 9 patient 1087 (Dead Sea, IL); lane 10 patient 1070 (Hemdat, IL); lane 11 patient 1088 (Negev, IL); lane 12 neg. FP. TaKaRa-Ex-Taq polymerase was used and 5 µl of BSA (10mg/ml) was added to each reaction. The filter paper samples were lysed in 200 µl NET-buffer, digested with proteinase K, 2 µl were used as template, annealing temperature was 50°C.

Figure 14: PCR with primers Uni21/Lmj4 on filter paper extractions



Lane 1-6 phenol-chloroform extracted, lane 7-10 crude experiment, lanes 11-17 controls. Lane 1 PGEM; lane 2 patient 1075 (IL); lane 3 patient 1077a (Arava, IL); lane 4 patient 1077b (Arava, IL); lane 5 patient 1083 (Dead Sea, IL); lane 6 positive extraction control, mouse liver infected with *L.donovani;* lane 7 patient 1077a crude 1µl; lane 8 mouse liver, crude 1 µl; lane 9 patient 1077a crude 1µl + 1µl BSA; lane 10 mouse liver crude 1 µl + 1µl BSA; lane 11 *L.donovani*; lane 12 *L.aethiopica*; lane 13 *L.major*; lane 14 *L.major*; lane 15 *L.tropica*; lane 16 ? ; lane 17 neg. PCR-control. (Annealing of 48°C)

3.2.4. Selected patient cases diagnosed with kinetoplast primers Uni21/Lmj4

Results obtained from dermal scrapings on filter paper

1. One patient suffered from a severe and prolonged infection of the nose (photo 1); he had a second lesion on his hand. He was a young man of 20 years coming from Hemdat, an Israeli settlement, located in a hilly area close to the Jordan Valley. The direct analysis from the skin scrapings on filter paper (1 of each lesion) revealed a band of 820 bp (Figure 13 lane 10, Table No. 6, patient 6) from one lesion, and two bands from the other lesion (680 bp and 820 bp, the latter band being weaker, not shown). The PCR performed on the culture some weeks later yielded a band of 680 bp . These contradictive results will be discussed in chapter 4.

2. A married couple suffered from multiple lesions (12, respectively 9) distributed all over the body (Photo 5). They came from Holon, an urban center close to Tel Aviv and claimed they had not travelled either inside or outside the country for at least one year. It was the first case of CL in this part of the country and the origin of infection could not be identified. There was only a vague hypothesis that a sandfly might have been trapped in the car of a neighbour, who was travelling frequently to the Jordan Valley. PCR diagnosis succeeded from tissue scrapings in the couple from Holon (patients 9 and 10 in Table 6). *L.major* was diagnosed (680 bp product). The smears were positive but cultures failed to grow.

3. A visiting researcher from Uzbekistan had been vaccinated with a viable *L.major* strain. A lesion had developed at the vaccination site, which had no tendency to heal even after several months and was forming also satellite lesions (Photo 4). Even though the cause of the infection was clear, a skin scraping was collected, to test if the PCR would in fact confirm the infection of

Leishmania major. The PCR confirmed *L.major* (patient 24 in Table 6; lane 13, Figure 12-PCR also from the culture).

4. Ear scrapings from *Psammomys* (No. 25 in Table 6, Figure 16) were examined and a product of 680 bp was found in all 3 individuals, thus confirming the expected species, *L.major*.

5. The positive extraction controls (infected mouse liver, No. 26 in Table 6) always yielded a 800 bp product, as expected for the *L.donovani* complex.

6. Patients 11 and 12 (Table 6) were diagnosed with *L.major,* and New World *Leishmania* species could be excluded as causative agent. The infections were therefore contracted in Israel and not during the journey in South America.

3.3. Genus specific amplification with kDNA primers 13A/13B:

All *Kinetoplastidae* (*Leishmania, Trypanosoma, Crithidia*) tested in the PCR with primers 13A/13B yielded a product of 120 bp. The amplification of the positive extraction control indicated successful DNA extraction. The sensitivity with a serial dilution of purified DNA was at 10 fg (data not shown) with ethidium bromide staining (Gel Star staining not needed). This confirmed the sensitivity found by Rodgers *et al*., (1990) who had introduced the primers and had found a sensitivity corresponding to far less than one parasite. The negative extraction controls (negative filter paper, human blood on filter paper) of the first few extraction series were contaminated. Only after potential sources of contamination were eradicated, by following strictly the precautions mentioned in chapter 2 (Material and Methods), the problem was controlled. At the time the contamination problems were controlled with the more sensitive, genus specific PCR the patient samples (which had been mainly used to establish the diagnosis with primers Uni21/Lmj4) were used up. Primers 13A/13B were evaluated with new sample groups, which were received later (eg. animal samples). For species specific identification some of the PCR-positive samples were submitted to PCR with primers Uni21/Lmj4. This succeeded with 3 out of the 4 tested *Psammomys* samples, and *L.major* was confirmed (Figure 16).

3.4. Studies on reservoir animals:

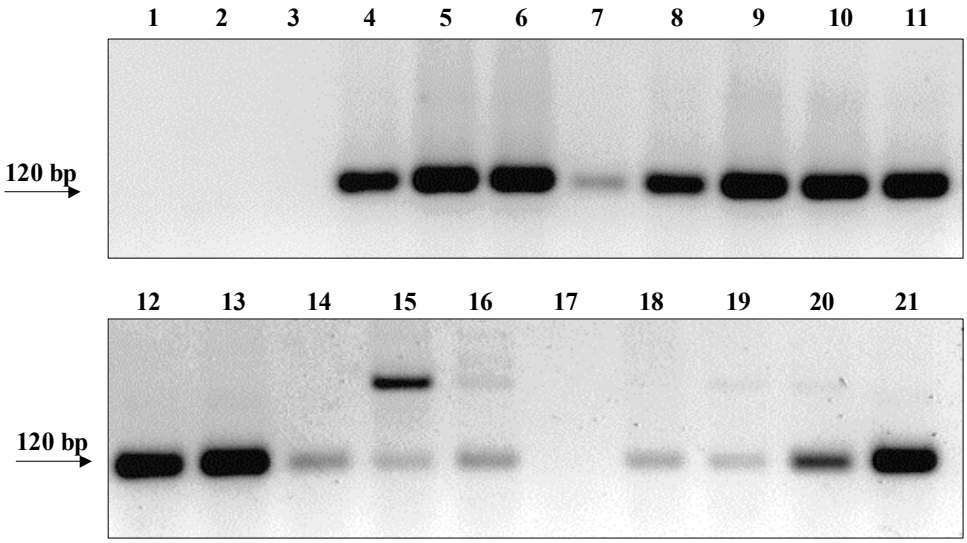
1. Desert rodents: (Photo 18)

The animal results are summarized in Table 7. As expected, the PCR proved to be more sensitive than microscopy on the *Psammomys* ear scrapings: Every microscopically positive smear was also positive by PCR. The genus specific PCR with primers 13A/13B detected *Leishmania* infections in 93.3 % (28 out of 30) of the tested *P.obesus* samples. Out of the 28 PCR-positive samples 20 were positive by microscopy (71.4%). Eight out of 10 smear-negative samples were thus positive by PCR. (Microscopy was performed by Gideon Wasserberg, Ben Gurion

University, Beer Sheva). Some of the smears were double checked in our laboratory, the microscopical results were in concordance between the two laboratories. The amplifications were highly efficient, as most samples showed intensive bands (Figure 15). The negative *Psammomys* control (laboratory animal from the Diabetes Unit, Hadassah Hospital) was always negative (as expected). As mentioned already *L.major* was confirmed with primers Uni21/Lmj4 (Figure 16) and later also with ITS-1 amplification and RFLP-analysis (Figure 17). In the latter experiment non human pathogenic *Leishmania* species (reference strains) were amplified and restricted for comparison.

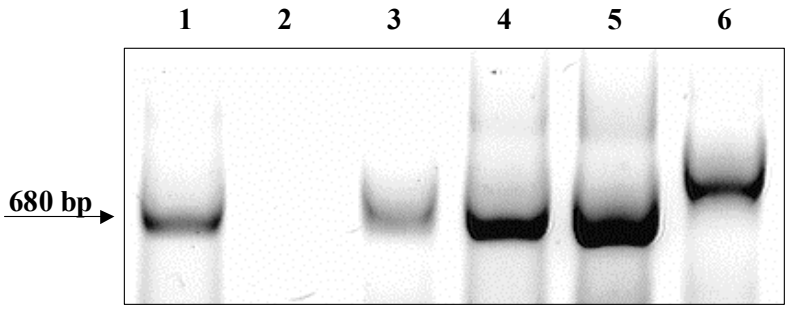
According to PCR, 65% (11 out of 17) ear scrapings from *Gerbillus dasyarus* were positive (Figure 15). The PCR products were seen as faint bands. A new host species was identified (discussed in chapter 4). After positive PCR results the smears were examined once more. One single amastigote was finally detected in one of the smears and approved by three examinators, thus confirming the findings by PCR. The faint bands of the PCR products correlated with the scarcity of amastigotes in the smears. This explains also why the *Gerbillus dasyarus* sample failed to amplify in the ITS-1 PCR (Figure 17). The larger band (Figure 15, lane 15) may be a non-specific amplification seen sometimes also in amplifications from dog samples. A reliable negative control of the same animal species was not available which could have helped to clarify this non-specific amplification.

Figure 15: *Psammomys obesus and Gerbillus dasyarus*
PCR with primers 13A/13B, Chelex extraxted ear scrapings on filter paper



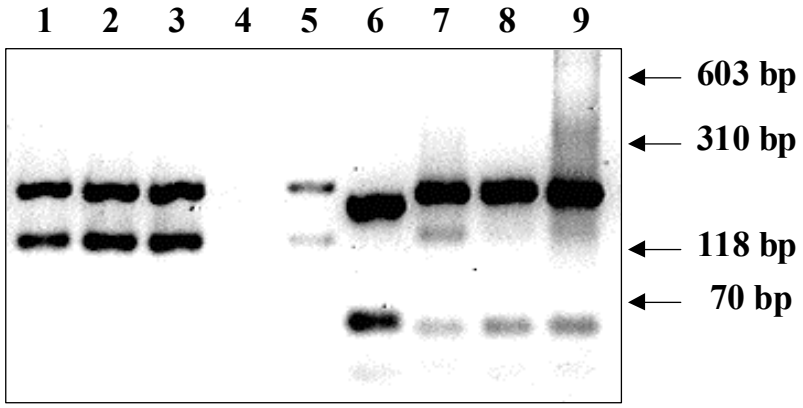
Lane 1 negative filter paper; lane 2 neg. blood; lane 3 negative *Psammomys* control; lane 4-13 *Psammomys* ear scrapings; lane 14-19 *G. dasuyrus*; lane 20 *L.major*; lane 21 *L.major*

Figure 16: *Psammomys* ear scrapings with primers Uni 21/ Lmj4:



Lane 1-4 *Psammomys*; lane 5 *L.major*; lane 6 *L.tropica*

Figure 17: PCR with ITS-1 primers and restriction with *BsuRI* (*HAE III):*



Lane 1-3 *P.obesus,* Qziot (IL); lane 4 *G.dasyarus*, Qziot (IL); lane 5 *L.major*; lane 6 *L.tropica*; lane 7 *L.arabica*; lane 8 *L.gerbilli*; lane 9 *L.turanica*.

2. Canids:

The 4 tested dogs (Photo 20) were positive by PCR with primers 13A/13B (results not shown). Interestingly, a repeated extraction and PCR performed by a collegue showed the same intensities of bands on the gel. This indicated a positive correlation between antigen load and the quantity of amplified product. The 21 jackal ears were all negative. The extraction and the PCR had been successful as shown by the positive extraction- and positive PCR-controls. The 14 extracted jackal spleens in formalin were PCR-negative too. In order to test, if the negative results were due to inhibition or poor DNA quality mammalian primers were tested on 3 of the jackal samples (by Carney Matheson, guest scientist at the Kuvin Center, University of Queensland, Australia). Faint signals were seen, meaning that amplification was principally possible from formalin fixed samples, but that this preservation method was probably not

suitable for sensitive diagnosis. It remains unclear, if any of the jackals were *Leishmania*-positive.

3. Hyrax:

All hyrax samples (parched and fresh tissue samples) were PCR-negative. A hyrax is shown in Photo 19.

Table 7: Results from animal samples:

species	Specimen	No. of samples	PCR +	smear +
Psammomys	ear scrapings	30	28	20
Gerbillus dasyarus	ear scrapings	17	11	1
Meriones crassus	ear scrapings	4	0	0
dogs with CVL	EDTA blood	4	4	n.a.
Jackal	ear biopsies, frozen	20	0	n.a.
Jackal	spleens in formalin	14	0	n.a.
Hyrax	nose, skin, bone	6	0	n.a.
Hyrax	ear biopsy, frozen	1	0	n.a.
mouse	liver tissue	positive control	always positive	positive
hamster	foot pad aspirate	1	positive	n.a.

CVL =canine visceral leishmaniasis
n.a. =not available

3.5. Specific detection of *Leishmania braziliensis* with kDNA primers MP3H/MPL1:

Results obtained from dermal scrapings preserved on filter paper:

It was important to establish PCR diagnosis also for *Leishmania* infections contracted in the New World. Since especially *L.braziliensis* infections are feared due to the risk of MCL the most important aim was to identify *L.braziliensis*. The PCR with primers MP3H/MPL1 was successfully employed on the reference strains as well as on the patient samples. All tested *Leishmania* species of the *L.braziliensis* complex (*L.braziliensis, L.guyanensis, L.panamanensis*) were amplified and yielded a product of 70 bp (in Figure 18 the band appears to be slightly larger, the band was usually exactly at 72 bp of the marker). *L.mexicana* and the *Leishmania* species of the Old World did not amplify, as expected. The method proved to be highly specific with the only exception that *L.amazonensis* (*L.mexicana* complex) was sometimes amplified with a very faint signal, which was not expected. A carry-over contamination between the New World reference DNA strains can not be excluded. The DNA had been handled by several different persons previously. Contamination could have occurred by pipetting the different species into PCR tubes for one experiment, keeping in mind that this PCR method is capable of detecting DNA of far less than one parasite (Lopez *et al.,* 1993). No other *L.amazonensis* strain was available. The sensitivity was known to be at least as high as with the genus specific primers 13A/13B, according to the literature (Belli *et al*., 1998). Serial dilutions with purified DNA give

generally only theoretical sensitivities, which are not necessarily reproduced with clinical samples. Therefore it was preferred to directly evaluate the sensitivity on the clinical samples, in comparison with the genus specific primers (13A/13B). The sensitivity was 6/6 (100%) of the patients who had visited Tuiji in Bolivia and who were suspected to be infected with *L.braziliensis*. The genus specific primers gave positive results in all 11 patients who were examined (4 patients infected in the Old World, 1 in Guatemala). The follow-up study (repeated PCR with the genus specific and the *L.braziliensis* specific primers directly after and months after treatment) indicated that the *L.braziliensis* specific primers MP3H/MPL1 were even slightly more sensitive than the genus specific primers 13A/13B (eg. patients 1, 2 in Table 8).

Patients of the Tel Hashomer Hospital:

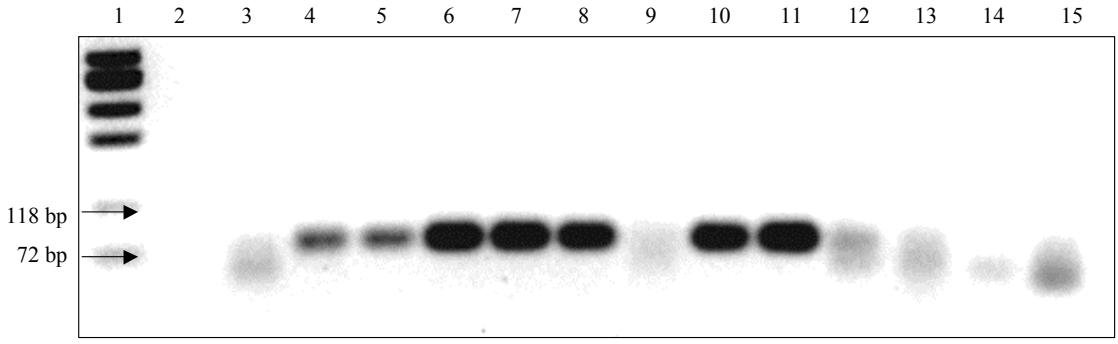
Skin scrapings and cultures were taken from 11 patients who were admitted to the Tel Hashomer Hospital (Tel Aviv) for systemic treatment with Pentostam. Species specific diagnosis especially of the *L.braziliensis* complex was required since 7 patients had contracted CL in the New World (Photos 15-17). Six patients had visited Tuiji, an attractive tourist site in the Bolivian rain forest, 1 patient had been travelling in Guatemala. The other 4 patients had been infected in the Old World, 1 patient in Turkmenistan, 1 patient in Afula (Galilee), 1 in Korazim, a village on the northern slope of the lake of Galilee and another patient had been infected in the Negev. The patient from Afula was suffering from a severe infection of the nose (Photo 2), the patient from Korazim suffered from a lesion, which involved one eyelid (Photo 3). Both cases were of special interest, since *L.tropica* was expected to be the causative agent, and both places had not been identified as being *L.tropica* foci previously. The diagnosis of CL had been made in all 11 patients beforehand in the hospital, by microscopy of skin biopsies. All 11 patients yielded positive results with the genus-specific primers 13A/13B. The results are specified together with the results of primers MP3H/MPL1 in Table 8.

The patient who was infected in Guatemala (patient 12) was negative with the *L.braziliensis* specific primers MP3H/MPL1 and positive with the genus specific primers 13A/13B, which was suggestive of an infection with *L.mexicana*. Patients 4, 5, 8 and 9 were infected in the Old World. All were positive with the genus specific primers and negative with the *L.braziliensis* specific primers, thus confirming the specificity of the primers.

Repeated PCR, directly after treatment with pentostam and also several months after completing the therapy, was still positive in most cases, though weaker. In some cases only one of the two PCR methods reproduced signals after repeated sampling (after treatment). Specific results are documented in Table No 8 and Figures 18-20. Cultures were positive in patients 1 (from 2

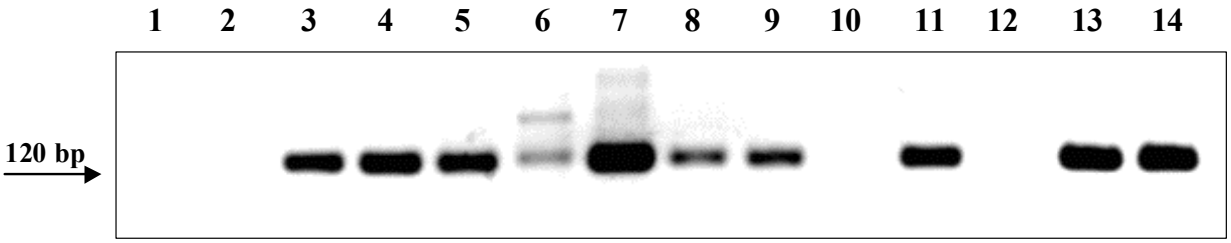
different lesions), 4, 7 and 9 (collected before treatment). This was the first time that *L.braziliensis* has been diagnosed in Israel.

Figure 18: *L.braziliensis* primers MP3H/MPL1:



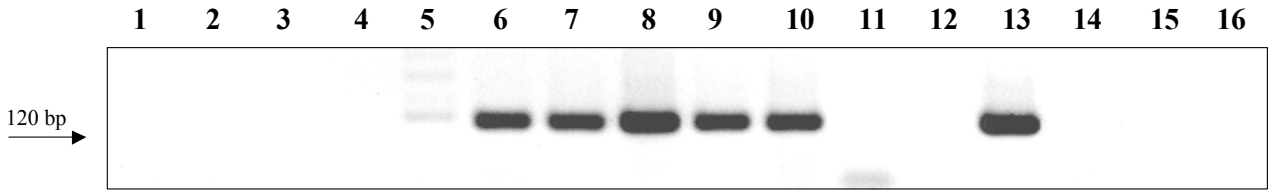
Dermal scrapings on filter paper, phenol-chloroform extraction. Lane 1 DNA size marker ϕHAE ; lane 2 neg. FP; lane 3 neg. blood; lane 4 patient 1a (BO), relapse?; lane 5 patient 1b (BO), relapse?; lane 6 patient 6a (BO) before treatment; lane 7 patient 6b (BO) before treatment; lane 8 patient 6a (BO) after treatment; lane 9 patient 4 Afula, IL); lane 10 *L.braziliensis*; lane 11 *L.guyanensis*; lane 12 *L.amazonensis* (contamination?); 13 *L. mexicana*; lane14 *L.major*; lane 15 neg. PCR-control

Figure 19: Genus specific PCR with primers 13A/13B (120 bp)



Patients 1, 2, 4 before treatment, patient 3 after systemic treatment with pentostam.
Dermal scrapings on filter paper, guanidine extracted. Lane 1 neg. FP; Lane 2 neg. blood; lane 3 patient 1a (BO); lane 4 patient 1b (BO), (taken from another lesion); lane 5 patient 2 (BO); lane 6 patient 3 (BO); lane 7 patient 4 (Afula, IL); lane 8 patient 1201 (IL); lane 9 patient 1203 (IL); lane 10 patient 1204 (IL); lane 11 patient 1187 (IL), lane 12 negative PCR-control, lane 13 *L.major*; lane 14 *L.tropica*.

Figure 20: Genus specific PCR with primers 13A/13B:



Patients 1-4 after treatment with pentostam. **Lane 1 negative filter paper; lane 2 negative blood, lane 3 patient 1 (BO); lane 4 patient 2 (BO); lane 5 patient 3 (BO); lane 6 patient 4 (Afula, IL); lane 7 and 8 patient 5a, 5b (Korazim, IL), (2 paraffin embedded skin biopsies taken from two lesions); lane 9 and 10 paraffin embedded skin biopsies from Tchechia, patient infected in Jordan; lane 11 Hyrax ear; lane 12 sandfly (West Bank); lane 13 *L.major*; lane 14 negative PCR control; lane 15 *L.mexicana*; lane 16 *L.braziliensis***

Table 8: Results of patients from the Tel Hashomer Hospital:

Patient Nr.	**1**	**2**	**3***	**4****	**5****	**6**
Origin of infection	Tuiji, Bolivia	Tuiji, Bolivia	Tuiji, Bolivia	Afula, IL	Korazim, IL	Tuiji, Bolivia
1st Sampling	8.5.00	8.5.00	8.5.00	8.5.00	1.6.00	11.7.00
1st PCR genus	+/+	+	+	+	+	+/+
1st PCR L.braz.	+/+	+	+	-	-	+/+
Culture	+/+	n.d.	n.d.	+	-	-
Identification	***L.braziliensis***	***L.braziliensis***	***L.braziliensis***	***L.tropica?***	***L.tropica?***	***L.braziliensis***
2nd sampling	1.6.00	1.6.00	1.6.00	1.6.00		5.9.00
2nd PCR genus	-	-	+ weak	+	+	+
2nd PCR L.braz.	+ weak	+ weak	+	-	-	++
3rd sampling	5.9.00 relapse?	10.10.00	26.12.00 relapse?	11.7.00 relapse?		10.10.00
3rd PCR genus	-	-		+		+
3rd PCR L.braz.	+ weak	-		-		+
4th sampling	relapse?		9.1.01			19.11.00
4th PCR genus	-/-/+ weak					+/- weak
4th PCR L.braz.	+/+/+		-			-/-
5th sampling	19.11.00 2ndtreatment					23.01.01
5th PCR genus	+/+ weak					
5th PCR L.braz.	-/-					+

Patient Nr.	**7**	**8 *****	**9*****	**10**	**11**
Origin of infection	Tuiji, Bolivia	Negev, IL	Turkmenistan	Tuiji, Bolivia	Guatemala
1st Sampling	23.10.00	12.11.00	12.11.00	21.11.00	10.10.00
1st PCR genus	n.d.	+/+	+/+/-	-/+/+ weak	+
1st PCR L.braz.	+	?	-/-/-	+/+/+ weak	-
Culture	+	-	+		
Identification	***L.braziliensis***	***L.major ?***	***L.major ?***	***L.braziliensis***	***L.mexicana?***
2nd sampling	19.10.00	12.11.00		24.12.00	
2nd PCR genus	+?	+/+		+	
2nd PCR L.braz.	-				
3rd sampling	19.11.00			march 2001	
3rd PCR genus	+ weak				
3rd PCR L.braz.	+ weak			+/+	
4th sampling	9.1.01				
4th PCR genus					
4th PCR L.braz.	+				
5th sampling	march /01				
5th PCR genus					
5th PCR L.braz.	+				

L.braz. =means *L.braziliensis.* *Patient 3 had already had treatment before the first visit. ? indicates that the species was suspected, according to the patient history and the results obtained so far. Species were

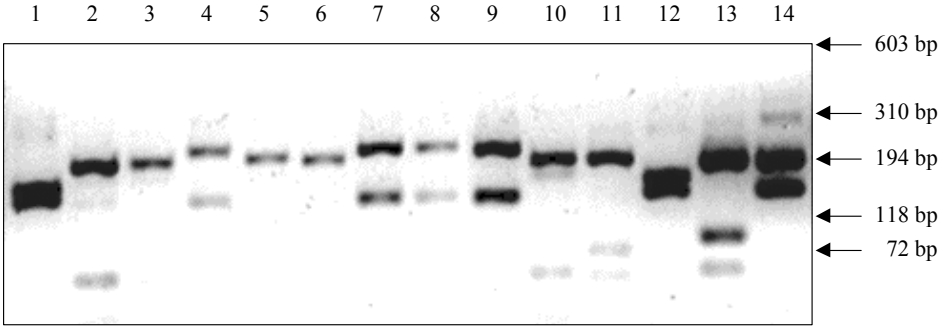
confirmed later with ITS-1 PCR: **Patient 4 and 5 later were confirmed with *L.tropica*,*Patients 8 and 9 were confirmed with *L.major*, patient 9 with kinetoplast primers Uni21/Lmj4 from the early culture.**

Figures 19 and 20 represent diagnostic PCR experiments (from dermal scrapings on filter paper) at two different times. Several patients are identical in both figures, with the difference that Figure 19 shows results before treatment and Figure 20 shows results after the 3 weaks course of treatment with pentostam: Bands in lanes 3 and 4 are derived from two different lesions of patient 1. Figure 20 shows that no amplification was seen after treatment in patient 1 (lane 3, only one lesion re-examined). The same was found in patient 2 (lane 5 Figure 19, lane 4 Figure 20). Patient 3 (lane 6 in Figure 19) was examined only after treatment and then re-examined later (lane 5 Figure 20). This patient had just completed the treatment when the sampling was performed for the first time. As seen in this patient and also in patient 4 (lane 7 in Figure 19, lane 6 in Figure 20) DNA was amplified also after the treatment, though weaker. This will be discussed in chapter 4. The other patients are not identical in the two figures, because it was naturally the case that new cases were added to the series whenever diagnosis was required in new patients visiting the outpatient clinic for travellers.

3.6. Amplification of the ITS-1 region and RFLP analysis:

All tested species (purified DNA of WHO reference strains) were amplified. The products had a size of about 300 bp, with minor differences seen between some of the species, eg. *L.braziliensis* products appeared to be slightly smaller (not shown). The sensitivity was up to 1 pg when tested with purified DNA. Out of 36 previously positive extractions (thawed for reuse in this PCR) 25 were successfully amplified and submitted to restriction with the enzyme *BsuRI.* The tested samples showed the restriction patterns as it was expected (species specific diagnosis had been made previously in most cases). Out of 5 patients from the Hadassah-Hospital 4 were infected with *L.major* and 1 patient with *L.tropica*. The recently discovered endemic sites Wadi Albethan (Photos 10, 11) and Korazim were confirmed as being *L.tropica* foci, as well as the infection seen in patient 4 from Afula (Galilee) (Table 8, Figure 21 lane 2). Some *L.tropica* amplifications have been comparatively weak, the smaller expected band at 57 bp is therefore not seen (in lanes 3 and 5, compared to lane 10, reference strain). Therefore these *L.tropica* results appear to be similar to the *L.d.infantum* reference (lane 11). The patients who were infected in Tuiji (Bolivia) were once more confirmed with an infection due to *L.braziliensis*. Table 9 shows the expected sizes of restriction fragments of each species and the results obtained from dermal scrapings on filter paper. Figure 21 shows one example of ITS-1 amplification and *BsuRI*-digestion.

Figure 21: PCR with ITS-1 primers and restriction with *BsuRI:*



Lane 1 patient 1 (BOL; lane 2 patient 4, Afula (IL); lane 3 patient 1201 (IL); lane 4 patient 1202 (IL); lane 5 patient 5, Korazim (IL); lane 6 hamster liver infected with *L.d.infantum*; lane 7 patient 1175 (IL); lane 8 patient 1168 (IL); lane 9 *L.major*; lane 10 *L.tropica*; lane 11 *L.d.infantum*; lane 12 *L.braziliensis*; lane 13 *L.mexicana*; lane 14 *L.amazonensis.*

Table 9: *BsuRI* restriction fragments of ITS-1 amplificates:

Species	**Fragments after Restriction with *BsuRI* (bp)**	**Results**
L.major	203 132	P1167 (IL) P1202 (IL) P1175 (IL) P1168 (IL) 3 *P.obesus*, Qziot (IL)
L.tropica	185 57 53 24	Wadi Albethan No.19 P 4 Afula (IL) P1201 (IL) P5 Korazim (IL)
L.donovani complex	184 72 54	hamster liver on fp infected with *L.donovani* (2x)
L.aethiopica	200 57 54 23	
L.braziliensis	156 143	P1 (BOL) (2x) P2 (BOL) P7 (BOL) P10 a/b (BOL)
L.guyanensis	156 137	

L..panamensis	156 139	
L.mexicana	186 88 59	
L..amazonensis	186 142	
L.turanica	203 54 52 24	

3.7. A new focus of *L.tropica* in Wadi Albethan, West Bank:

One major purpose of the project was the study of the endemic sites in the West Bank, since most of them were not sufficiently understood and some of them were not documented yet. A new *L.tropica* focus has been identified in the country, in an Arab village named Wadi Albethan. The area of Wadi Albethan is mountainous, declining towards the Jordan Valley (Photos 10 and 11). Wadis (dry riverbeds) are connecting with the Jordan Valley. A brook is running through the village. In the center of the village settlement is dense, in the periphery houses are more isolated, surrounded by meadows with various kinds of trees and bushes (predominantly almond trees). Animals (predominantly goats) are kept in open sheds. The inhabitants claimed the infections only occurred for about one year. The characteristic lesions were not known before to the local population. Nevertheless, one young man had been suffering from leishmaniasis recidivans (lupoid leishmaniasis) for 9 years (Photo 8). He presented with a scarring lesion on his cheek, with active eruptions on the border of the lesion, and with one separate lesion on the nose. Another youth had an old scar (Photo 9). Interestingly, the inhabitants reported that hyraxes (Photo 19) had become abundant over the past two years. Due to the altitude of this area a focus of *L.tropica* was suspected. Several visits to this village were undertaken. In some houses several family members suffered from cutaneous lesions, some of them had multiple lesions (Photo 6). The lesions had appeared between 1 to 4 months before, in one person the lesion had evolved earlier (8 months before). Adults and children were equally affected. The severity of the lesions ranged from small papules to extensive and deep ulcers. Ulcers located on the nose tended to be severe. Some of the ulcerative lesions were obviously super-infected with bacteria (Photo 7). Some of the patients had received treatment with either liquid nitrogen or intralesional pentostam. Some infected residents had not seen a doctor and were left untreated. A visit to a doctor and subsequent treatment appeared to depend on the income of the family. Some families could not afford medical care. The information presented here is based on observations made during sporadic visits; a systematical epidemiological survey has not been carried out yet.

The preservation of skin scrapings on filter paper proved to be a suitable preservation method for PCR-diagnosis in this field study. The PCR with primers 13A/13B succeeded at first in 12 out of 23 guanidine extracted samples. This provided a very good result, considering that the methods were applied for the first time in a real field study in an unknown focus. A second extraction and re-amplification using only 2 µl template instead of 10 µl showed that all patients were positive, indicating inhibition by some components in the DNA-extract (the repeat was performed by Kefaya Azmi, Al-Quds-University). Primers Uni21/Lmj4 failed to directly prove the species, but later *L.tropica* was confirmed in one tested sample by ITS-1 amplification (Figure 20, Table 9) and *BsuRI*-digestion. *L.tropica* was also confirmed by other methods based on the successfully cultured strains (EF, Lionel Schnur and PPIP-PCR, Carol Eisenberger). The microscopical examination of the smears of 23 patients from Wadi Albethan revealed amastigotes in 9 smears, which is a sensitivity of 39%. Four smears had to be classified as negative, even though the microscopical picture was highly suggestive of a leishmanial infection: structures in the size of amastigotes were seen in groups either intracellular in macrophages or extracellular, but no nuclei and kinetoplasts could be identified. The following table shows epidemiological data and diagnostic results of the examined residents of Wadi Albethan.

Table 10: Results from patients from Wadi Albethan:

No.	Sex	Age	Duration of lesions in months	Number of lesions	PCR 13A/13B	Culture	Smear	Therapy?
1	f	~12	2	2	+	n.d.	++	
2	f	~11	1	1	+	n.d.	++	
3	f	13	1	1	+	-	-	1 P
4	f	10	1	1	+	-	-	1 P
5	m	25	8	2	+	-	-	no
6	m	48	3	6	+	-	-	N
7	f	12	1	1	+	-	-	N
8	f	35	2	4	+	n.d.	+	N
9	m	13	4	3	+	-	-	3 P
10	m	50	3	2	+	-	-	4 P
11	m	3	4	1	+	n.d.	+	1 P
12	m	3	3 weeks	1	+	n.d.	-	no
13	m	23	2	4	+	n.d.	++	no
14	m	43	4	3	+	n.d.	-	4 P
15	m	27	1	1	+	-	+	no
16	m	15	1	1	+	-	++	?
17	m	44	4	5	+	+	+	2 P
18	m	19	3	1	+	n.d	-	2 N
19	f	18	1	1	+	n.d	-	no
20	m	4	3	3	+	n.d	-	3 P
21	m	1	3	1	+	n.d	+	?

22	m	4	1	3	+	n.d	-	no
23	f	23	1	7	+	+	-	1 P

f =female; m =male; n.d. =not done; contam. =culture contaminated with bacteria; P =intralesional pentostam; N =liquid nitrogen. The number refers to the number of doses received; no =no treatment received

3.8. The aims of the study have been achieved:

1. Direct PCR diagnosis has been established in the laboratory and is ready to be used in clinical laboratories. The tested sampling procedure (dermal scrapings on filter paper) proved to be a highly suitable method which can be recommended for future applications. Diagnosis of CVL(VL) is available too, using peripheral blood spots on filter paper. Many observations have been made, related to preservation, extraction and amplification. This may help to select methods according to different purposes. Extraction methods have been simplified with regard to the application in clinical laboratories (eg. Chelex extraction).
2. 2. The differentiation of *L.major* and *L.tropica* has been achieved. Primers Uni21/Lmj4 were able to make the distinction but the diagnosis from dermal scrapings did not always succeed. With the recent introduction of the ITS-1 primers a sensitive system was established for the differentiation of nearly all species. The latter is also able to distinguish *L.d.infantum* infections, the third endemic species in the country.
3. PCR diagnosis directly from lesions has been established also for the *L.braziliensis* complex specifically, using kinetoplast primers MP3H/MPL1. This can help to decide if patients with New World leishmaniasis need to be hospitalized for 3 weeks or not. Species-specific diagnosis of the New World *Leishmania* species had not been available in the country before. The introduction of the ITS-1 method improved the quality of diagnosis, because it could also positively identify the *L.mexicana* complex (including the distinction of *L.mexicana* and *L.amazonensis*).
4. It has been shown that the methods employed (DNA extraction and PCR) were suitable for screening of reservoir animals. PCR was far more sensitive than microscopy, which clearly justified the method for diagnosis and for epidemiological studies.

   In addition, some important results have been obtained: A new host species has been discovered (*Gerbillus dasyarus*) and a new focus of *L.tropica* has been identified (Wadi Albethan, West Bank).

Photography:

L.tropica infections which required systemic treatment with pentostam

Photo 1
L.tropica, Hemdat, IL

Photo 2
L.tropica
Afula, Galilee

Photo 3
L.tropica
Korazim, Galilee

Cutaneous leishmaniasis by *L.major*

Photo 4
Lesion with satellite, complication of vaccination with a *L.major* strain in scientist from Uzbekistan

Photo 5
Mutiple lesions by *L.major* in Israeli patient from Holon, near Tel Aviv

Wadi Albethan, West Bank (Samaria), outbreak of *L.tropica*:

Photo 6
multiple lesions

Photo 7
Superinfected lesion

Photo 8
Leishmaniasis recidivans
(lupoid leishmaniasis)
for 9 years

Wadi Albethan, West Bank (Samaria), outbreak of *L.tropica*:

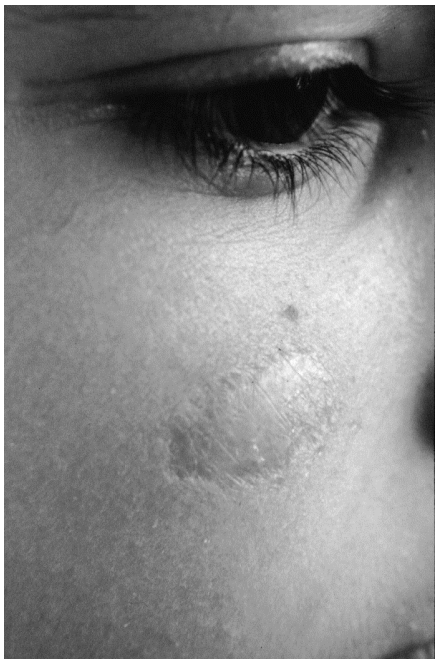


Photo 9
CL scar

Photo 10
Wadi Albethan,
view to the east,
towards the Jordan
Valley (behind
mountain range)

Photo 11
Area of high incidence
in Wadi Albethan

Endemic sites of Cutaneous and Visceral Leishmaniasis

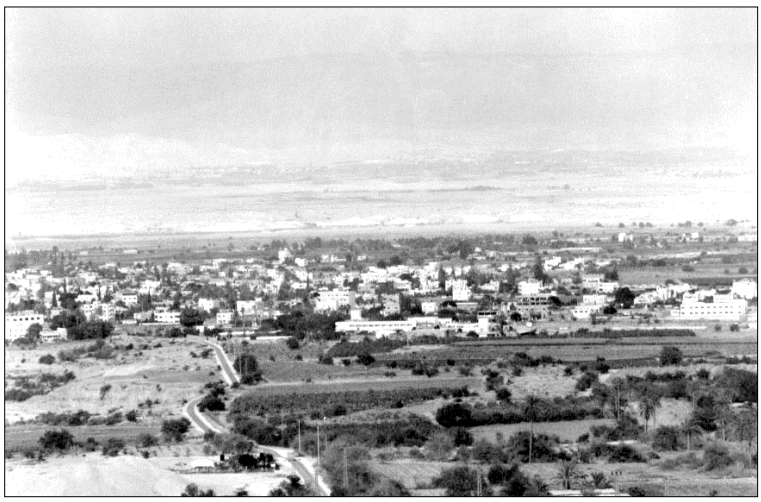


Photo 12
Jericho,
Jordan Valley
L.major

Photo 13
Kfar Adumim,
Judean Desert
L.tropica

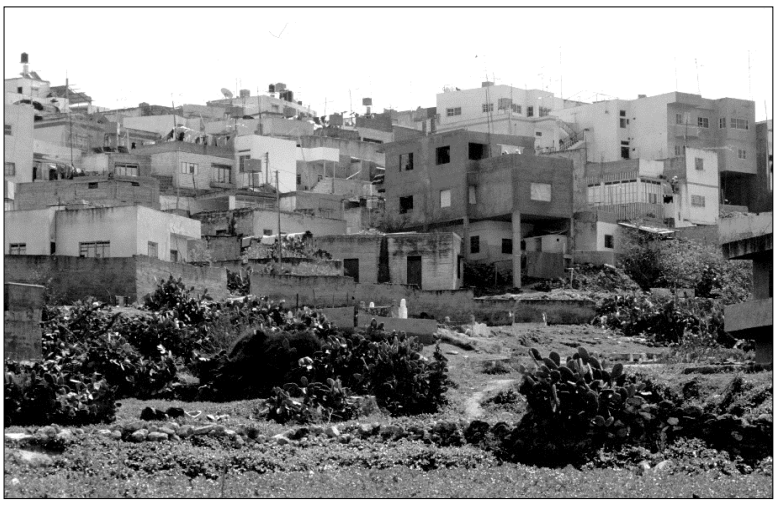


Photo 14
Jenin district,
West Bank,
L.d.infantum

L.braziliensis infections in Israelis who were infected in the Bolivian rainforest

Photo 15
L.braziliensis,
before treatment
with pentostam

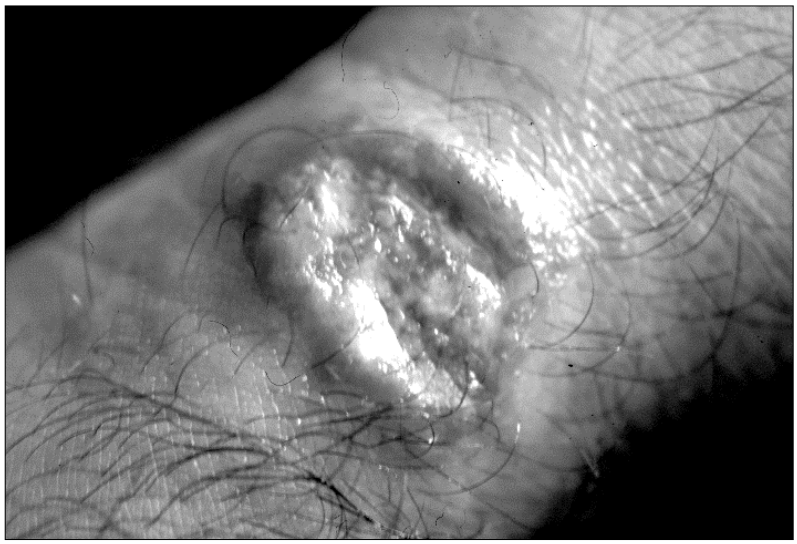


Photo 16
L.braziliensis,
before treatment
with pentostam

Photo 17
L.braziliensis
After treatment
with pentostam

Reservoir animals

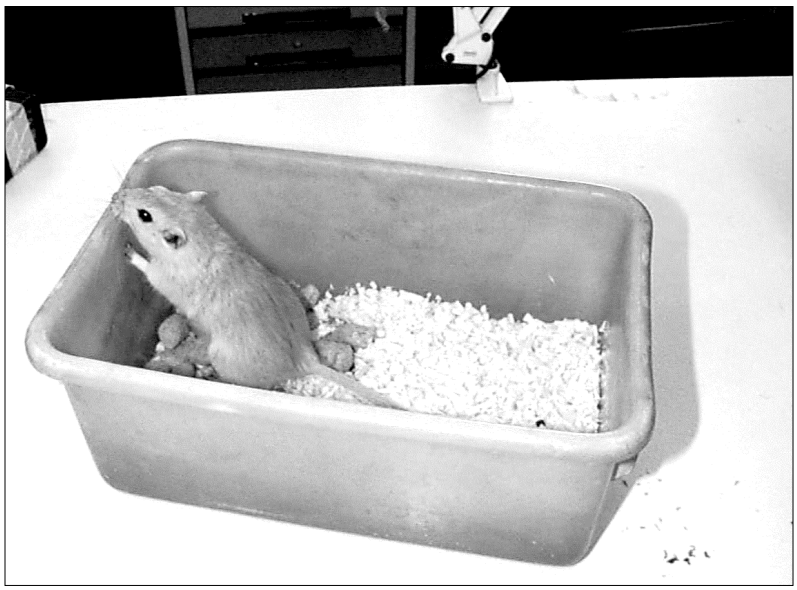


Photo 18
Psammomys obesus,
(Fat sand rat)
desert rodent,
reservoir of *L.major*

Photo 19
Hyrax, En Gedi,
suspected reservoir
of *L.tropica*

Photo 20
Dog with CVL,
Nataf, infected with
L.d.infantum

4. Discussion

The achievement of this research has been the selection of appropriate methods for the specific requirements of the Middle East. Many others have worked on the improvement of PCR-diagnosis of leishmaniasis in other regions of the world, and much has been achieved in the past decade. Based on the experiences made within this research, different aspects of extraction- and PCR-methods will be discussed, which can be used as a basis for future applications.

The PCR methods are discussed chronologically, as they have been gradually established in the laboratory. The results are mainly discussed within the sections of the PCR-methods by which they were obtained. This proceeding was favoured because it reflects the actual situation as it was, where neither the methods nor the samples were available at the beginning.

The collection of samples depended greatly on the contacts made during the study. Sometimes coincidences led to the collection of new sample groups, as was the case with the new *L.tropica* focus in the West Bank (Wadi Albethan): A dermatologist in Nablus had invited us to visit. The same day he had treated a patient with CL from Wadi Albethan. Through the initiative and help of Samir Sawalha (Masters student of the Al Najar University, Nablus), the first visit to families in the village was arranged immediately and more visits followed.

4.1. Sampling and preservation:

4.1.1. Collection of dermal scrapings:

The detection rate of leishmanial DNA by PCR in dermal scrapings proved to be higher than in microscopical smears, as especially seen in two study groups (desert rodents and patients from Wadi Albethan) The detection rate of PCR exceeded the detection rate of microscopy on dermal scrapings and skin biopsies also in previous studies (Belli *et al*., 1998; 1999; Rodriguez *et al.*, 1994). It is important that the sampling be performed correctly, according to the instructions given by Herwaldt, (1999) (chapter 2.2.1.). Belli *et al*., (1998) found the PCR on dermal scrapings (Chelex-extracted) to be 100% sensitive and also to 100% specific. Since dermal scrapings are less invasive and much easier to collect, he suggests that they can fully replace skin biopsies. This was also maintained by Rodgers *et al*., (1990), who suggested that patients should be spared from skin biopsies, especially if lesions are located in the face. Instead of sterile surgical blades, sterile wooden toothpicks can be used as well for sampling (Belli *et al*., 1998; Harris *et al*., 1998). It is known that skin biopsies have a higher detection rate than dermal scrapings if examined microscopically (Andresen *et al*., 1996), but with PCR the sensitivity is high enough using dermal scrapings.

4.1.2. Specimens other than dermal scrapings for direct PCR-diagnosis:

Apart from dermal scrapings, peripheral blood has been successfully used for diagnosis in this study. Others have previously shown that many different kinds of specimens can be used for PCR-diagnosis of leishmaniasis. Extracts from tissue samples, smears, lymph node and bone marrow aspirates and peripheral blood have been used successfully (Smyth *et al.* 1992; Lopez *et al.*, 1993; Eresh *et al.,* 1994; Wilson *et al.,* 1995; Laskay *et al.,* 1995; Bhattacharyya *et al.,* 1996; Osman *et al*., 1997; Belli *et al.,* 1998; Noyes *et al.* 1998). PCR has also been performed on sandflies (Rodriguez *et al*., 1999; Aransay *et al*., 2000).

4.1.3. Preservation on filter paper:

Blotting of tissue scrapings on filter paper is an excellent method for long-term preservation. The preservation on filter paper proved to be especially suitable for field studies due to very good conservation of the DNA, without cooling, with little storage space and low expenses. No additives for the preservation are required, which could potentially interfere with the PCR. The samples can be delivered to the laboratory weeks after collection without any loss of quality. Filter paper has been used previously for the preservation of peripheral blood spots (Osman *et al*., 1997; Campino *et al*., 2000), dermal scrapings (Harris *et al*., 1998; Färnert *et al*., 1999) and different kinds of aspirates (El Tai *et al*., 2000).

It has been shown in this study that the filter paper samples were stable for more than one year. The rodent samples had been stored at room temperature for more than one year before they were processed. The amplifications have been very efficient and no loss of quality was observed. A comparative study on *Leishmania* positive tissue on filter paper, which had been stored at different temperatures (-20°C, 4°C, 20°C) for more than one year, has shown that the DNA was conserved equally well at all temperatures. It can be assumed that humidity affects the stability of the DNA, but this has not been examined here. Freezing of filter paper samples may be preferred in humid climates. (Campino *et al*., 2000). Färnert *et al.*, (1999) have found that blood samples stored at 4° and at 20° C lost some sensitivity after a period of 30 days, whereas blood samples on filter paper were stable for several months at 4° and at 20° C. For the storage of blood samples the choice of anticoagulant is relevant. Färnert *et al.*, (1999) found inhibition in samples extracted from heparin-blood and equally efficient amplification from EDTA- and citrate-blood. Heparin is known to inhibit PCR. EDTA is also known to inhibit PCR, but the amount used for anticoagulation is small enough not to interfere with the reaction. By using filter paper also for peripheral blood (diagnosis of VL), the problem of PCR inhibition by anticoagulatory agents can be avoided.

4.1.4. Classical preservation methods:

It was demonstrated that samples differently preserved can be processed as well: Paraffin embedded biopsies (after deparaffinization, see passage 2.2.4.2.) and Giemsa-stained smears were extracted with the phenol-chloroform extraction and successfully amplified. An eventual loss of sensitivity can not be excluded but was not further examined. The formalin-fixed jackal spleens were all negative, either due to true negativity, to DNA degradation prior to the extraction or to inhibition. The biopsied material had not been preserved well, judging from the outer appearance. Formalin-fixed biopsies have been used previously for the diagnosis of leishmaniasis with good success by Mimori *et al*., (1998). The positive results with paraffin embedded biopsies and Giemsa-stained smears in this study show that samples other than dermal skin scrapings may also be proceeded (if received from other hospitals). This shows that the same methods can be employed for retrospective studies (Laskay *et al*., 1995).

4.2. Discussion of DNA extraction methods:

Several different extraction methods have been employed, ranging from thorough purification to very simple and crude methods. All extraction methods were successful, each of them having advantages and disadvantages. Two objectives had to be pursued, one being the best possible purification with elimination of inhibitory factors, the other being the highest possible yield of DNA. A good balance had to be found between the two requirements. Thorough purification was achieved only through many single steps, always implying a loss of template to a certain extent and an increased risk of sample cross-contamination. Crude extractions did not eliminate inhibiting factors sufficiently (additives can circumvent the problem). Besides, it was important to develop a method, which would readily be accepted in terms of practicability and cost effectiveness (time and material) in a clinical laboratory. Avoidance of hazardous chemicals is another criteria, which might be considered for routine diagnosis.

4.2.1. Phenol-chloroform-extraction:

The phenol-chloroform extraction is a very efficient and thorough purification method, which is comparatively hazardous and time consuming. Many steps of lysis, digestion, extraction and washing require much opening and closing of the 1.5 ml tubes, implying an increased risk of sample cross-contamination. The phenol-chloroform extraction is highly efficient in removing inhibiting factors, such as hemoglobin. It therefore proved to be reliable also for samples with a higher blood content. All samples that were conserved differently (formalin-fixed, paraffin-embedded biopsies and Giemsa stained smears) were proceeded with this method because it had the highest potential to remove inhibitory substances. Apart from that, phenol inactivates other

infective agents (eg. HIV), which may be a major consideration in some regions (eg. south-western Europe). Proteinase-K digestion with subsequent phenol-chloroform extraction is the best known classical extraction method, which was successfully employed also in many previous studies on clinical samples (Smyth *et al*., 1992; Ashford *et al*., 1995; Andresen *et al*., 1996; Osman *et al*., 1997; Mimori *et al*., 1998; Färnert *et al*., 1999; Campino *et al*., 2000).

4.2.2. Guanidine-extraction:

The guanidine extraction was efficient and reliable as well. It is especially efficient in the recovery of traces of DNA. The guanidine extraction proved to be less reliable on samples with a high blood content. The procedure is shorter and less steps are required than with the phenol-chloroform-extraction. The centrifugation steps are short (seconds) and a micro-centrifuge suffices. An advantage is also that the reagents are less hazardous than the ones used for the phenol-chloroform extraction. Besides, the samples are only once transferred to fresh 1.5 ml tubes, in contrast to 4 sets of fresh tubes needed during the phenol-chloroform extraction (contamination risk!). The guanidine extraction is a classical method in molecular biology, especially for forensic applications. Only few groups have employed this method in the field of leishmaniasis, also with good results (Noyes *et al*., 1998). An interesting study has been conducted by Lachaud *et al*., (2001) who compared the phenol-chloroform extraction with the guanidine extraction. In contradiction they found that the guanidine method was slightly more sensitive when whole blood (!) was used, whereas the phenol-chloroform method was superior when buffy coats were extracted. The difference was mainly that the phenol-chloroform extracted buffy coats produced stronger signals, the sensitivity was practically the same.

4.2.3. Chelex-extraction:

The Chelex-extraction was by far the shortest procedure. After over night digestion, heating for 10 minutes and a short centrifugation the supernatant was directly submitted to PCR. Results were obtained already in the early afternoon of the following day. Using the more thorough extraction methods, PCR-results could only be obtained by the evening of the day after the over night incubation. Furthermore, it was shown that an incubation of only 1 hour is sufficient for successful amplification, but the yield might not be maximal. The fact that the samples stay in the same Chelex-suspension from the beginning to the end of the procedure, without opening and closing of the tubes, reduces the risk of sample cross-contamination. Besides, no material is lost during the procedure as it is the case with the phenol-chloroform extraction, in which a small part of the original sample is naturally lost in every transfer of the upper phase. Other experiments with crude extractions have clearly indicated that hemoglobin residues may inhibit

the PCR. A relatively high tissue content and a low blood content (= sample quality with highest success rate) of the skin scrapings reduces the risk of inhibition. The results on the *Psammomys* ear scrapings have proved the high efficiency of this method, as it has been found as well in previous studies on human samples (Harris *et. al*., 1998; Belli *et. al*., 1998; 1999). An extensive study on dermal scrapings in 232 patients with American CL has shown that the Chelex extraction was suitable for reliable PCR diagnosis. Using primers 13A/13B, 205 patients were PCR-positive (85%) (Belli *et al*., 1999). Especially for larger studies, the Chelex extraction is a very convenient and time saving method. The superior qualities of the Chelex method are: low risk of contamination, saving of labour and material, combined with good sensitivity. For routine use in a clinical laboratory, the employment of the Chelex method could reduce the costs for the PCR-diagnosis of leishmaniasis enormeously. Several hours of bench work can be avoided and laboratory supplies can be spared (pipette tips, 1.5 ml tubes and chemicals). The Chelex method is limited in its capacity if inhibitory substances are present (has to be evaluated yet). For the diagnosis of VL it can be suggested to use the buffy coat (also spotted on filterpaper).

4.2.4. Crude extraction methods:

It has been shown that proteinase-K digestion and boiling (5 minutes) in a water bath was sufficient for successful PCR-amplification from dermal scrapings. These experiments were performed in the beginning of the study, when primers Uni21/Lmj4 were tested for their sensitivity on clinical samples. The fact that results were obtained with the least sensitive primers employed in this study is promising for diagnosis with the more sensitive primers. After the Chelex extraction was found to work well, experiments on other crude preparations became obsolete for this study. Crude DNA-preparations have been tested previously by others, also with good success (Rodriguez *et. al*., 1994; Laskay *et al.*, 1995; Harris *et al*., 1998; Belli *et al*. 1998). There are more variations and combinations, which would be worth-while to test, as for example proteinase-K digestion in 5% Chelex. Lopez *et al*., (1993) have used DNAse I digestion prior to PCR in order to expose more kDNA minicircles of the catenated network. The sensitivity was clearly increased when compared to non-digested samples (not tested in this study).

4.2.5. Extraction methods related to different types of specimens:

It was repeatedly observed that the quality of the results appeared to depend on the extraction method chosen for a certain type of sample. In a cooperative study (Mattheson and Anders, submitted 2001) a series of 7 different specimens were submitted to several different extraction methods in order to find the optimum extraction method for each different type of sample. Primers specific for the cytochrome b gene of the mitochondrial DNA were employed to detect

mammalian DNA. As expected, different extraction methods performed differently, depending on the types of specimens. The phenol-chloroform extraction was superior with the blood containing samples (1 drop of whole blood and of EDTA blood on filter paper, 500 µl of whole blood). The guanidine extraction was more efficient than the phenol-chloroform extraction with macerated raw tissue. The chelex method was almost as efficient as the phenol-chloroform extraction with blood spots on filter paper but it was much less efficient with larger amounts of blood and raw tissue. This comparative study showed that it is worth-while to select the extraction method according to the amount and type of sample.

4.2.6. Storage of extracted samples:

The extracted DNA was usually resuspended in ddH_2O. The samples were directly submitted to PCR for best results. At 4°C the DNA degraded within days. Therefore aliquots of the extracted DNA were stored at -20°C, in case additional reactions were necessary. Usually good results were obtained also after one round of freezing and thawing. It was considered to use TE buffer (pH 8) instead of ddH_2O, in order to stabilize the DNA. It was not used here because only small amounts of template would have been tolerated in the PCR-reaction due to the inhibitory activity of the EDTA present in the buffer. Since the phenol-chloroform extracted samples were resuspended in a much smaller volume than the guanidine- and Chelex-extracted samples, TE could be used for the phenol-chloroform extracted samples without any problems (advantage). The DNA is evidently more concentrated and less amounts of template are required for successful amplification, the inhibitory activity of EDTA is negligible then. When a small amount of TE-buffer (eg. 5µl) was added to extracted samples the PCR was not inhibited but the possibly higher stability of the DNA has not been examined yet.

4.3. Reliability of the employed PCR-methods:

4.3.1. Sensitivity:

The kinetoplast specific primers have been selected because of their potential for high sensitivity. The aim of the direct PCR diagnosis was the detection of single parasites from clinical material. Studies on purified DNA of reference strains have confirmed the high sensitivity of primers 13A/13B and MPL1/MP3H up to less than single parasites (Rodgers *et al*., 1990; Lopez *et al*., 1993). Experiments with serial dilutions of DNA within this study have found sensitivities with primers 13A/13B up to 10 fg, primers Uni21/Lmj4 up to 1 pg (25 fg DNA with Gels Star staining) and up to 1 pg with primers LITSRn/L5.8S (ITS-1). Even though primers Uni21/Lmj4 were theoretically capable of detecting single parasites it was often not seen. When clinical samples are examined less sensitivity is expected due to inhibitory agents.

Rodgers *et al*., (1990) found the sensitivity to be 10-100 fold less with clinical samples, compared to purified DNA from cultured strains (inhibition). Similar observations have been made repeatedly during this study. This fact could not be evaluated in a quantitative way since the intracellular amastigotes of a clinical sample would have to be compared with defined amounts of purified DNA which is practically impossible. A comparison between purified DNA and a defined number of promastigotes in peripheral blood is certainly the best possible comparative approach as recently performed by Dinse *et al*., (2001). The factor of cell lysis necessary to release the intracellular amastigotes in a real sample is not considered. Furthermore, dermal scrapings can not be standardized, every new sample has a different consistency (eg. amount of hemoglobin), which greatly influences the outcome. In order to assess the quality of the PCR in a more realistic way, results were compared with the results of the corresponding smears. The study with primers 13A/13B on desert rodents (Chelex-extracted) has clearly demonstrated a sensitivity which is unlikely to be surpassed. Primers MPL1/MP3H amplified even a smaller part of the kinetoplast minicircles (70 bp) and were therefore expected to have a similar sensitivity on clinical samples as primers 13A/13B. This was confirmed in the 6 patients who had contracted CL in Tuiji (Bolivia). The ITS-1 primers LITSRn/L5.8S were found to be highly sensitive on clinical samples since they were able to amplify many samples, which had been positive by primers 13A/13B and/or MPL1/MP3H earlier. This can be stated since the samples had been extracted earlier and had to be defreezed for this PCR. Freezing may lead to deterioration of the DNA quality.

In other studies, the sensitivity of PCR was always superior to the sensitivity of microscopy (Belli *et al*., 1998; Andresen *et al*., 1996; Rodriguez *et al*., 1994). PCR was also found to be more sensitive (100%) than serology (63%) (Ashford *et al*., 1997). It has been repeatedly shown that the potential sensitivity of PCR reached the detection of single parasites (Rodgers *et al*., 1990; Lopez *et al*., 1993; Noyes *et al*., 1998; Harris *et al*., 1998).

4.3.2. False negatives/ inhibition:

It was observed repeatedly that an increase of template (DNA, lysate or supernatant) in the reaction mix did not necessarily lead to a better result. On the contrary, it became obvious that beyond a certain amount of template DNA the amplification was inhibited. This applied also for well purified samples. In the first attempt to apply the direct PCR diagnosis on the 23 patient samples from Wadi Albethan 10 µl of template (10 µl out of 100 µl extracted, resuspended DNA) were used resulting in only 12 positives. In the repeat PCR (performed by Kefaya Azmi) only 2 µl were used resulting in 23 positives (100%). Several experiments showed that the inhibition can be controlled by adding either BSA (eg. 5 µl of a 10mg/ml solution) or DMSO

(2.5%) together with formamid (1%) to the reaction. Using these additives, the PCR tolerates more µl of template DNA, which is of interest especially for the less sensitive PCR (Uni21/Lmj4). Inhibition especially with crude samples was observed by others as well. Belli *et al*., (1998) circumvented the problem by diluting the samples up to 10-fold. Since the employed primers (13A/13B and MP3H/MPL1) were highly sensitive, the template in the diluted samples sufficed. With sensitive primers a small amount of template DNA (1-2 µl) is sufficient to ensure amplification.

The volume of the resuspension is also a factor worth to consider: A smaller volume is of advantage because the extracted DNA is more concentrated and therefore more stable. Only with the phenol-chloroform extraction could the volume be reduced to 25 µl. With the other methods the volume could not be reduced for technical reasons. The guanidine extracted samples were resuspended in a higher volume (100 µl) to allow the silica beads to be completely in suspension, in order to release the attached DNA into the solution. The Chelex extraction required at least 200 µl of 5% Chelex, otherwise the filter paper would not have been covered completely during the incubation.

False negative samples could be also due to uneven distribution of *Leishmania* DNA in an extracted patient sample, eg. if a sample contains only 1-10 parasites not every single µl of the resuspended DNA contains necessarily enough template to support amplification. It can be considered to amplify different amounts of one sample at the same time to avoid false negativity. Another possibility is to include an inhibition control, using primers which target human DNA. Human DNA is present in excess in all clinical samples and PCR-amplification can thus be controlled.

4.3.3. False positives/ contamination:

The main problem of sensitive PCR methods is the risk of contamination, leading to false positive results. Contamination was monitored by including at least two negative controls in every extraction series. A piece of plain filter paper was used also as a control of the filter paper itself. A human blood sample on filter paper was used to rule out any non-specific amplification of human DNA. For the *Psammomys* samples ear tissue was used from a negative laboratory animal. The amplification was controlled for any contamination related to the PCR itself (reaction mix without template).

It appeared that contamination occurred mostly during the extraction, with negative extraction controls being positive and negative PCR controls being negative. For this reason several series of extractions (Hadassah patients) had to be excluded from the study. The risk of sample cross-contamination was minimized, by following a number of precautions: Bleaching and UV-

radiating of surfaces and pipettes were the most important measures to avoid contamination. After these measures were followed strictly, the problem was controlled and extraction controls as well as PCR controls were then negative. The risk of contamination is known to be the main problem of PCR, which has been discussed also by others in relation to PCR-diagnosis of leishmaniasis (Adhya *et al*., 1995; Wilson , 1995; Belli *et al*., 1998).

4.3.4. Reproducibility of PCR-results:

In repeated extraction and PCR (13A/13B) from single drops of the same blood samples (4 dogs with CVL) the same intensities of bands were reproduced. This concordance suggested a high correlation between the parasitic load and the amount of amplified product. A positive correlation between the intensity of bands and the number of parasites seen by microscopy was also found by Andresen *et al*., (1996). The distribution of amastigotes within a lesion is not homogenous. One experiment with three dermal scrapings which had been collected as a series from three different areas of the same lesion, showed great differences in the intensities of amplified bands. It is therefore also possible that the smear contains amastigotes and the filter paper not, or reversely. Also the relative amount of blood influences the results. Bloody samples often contain less parasites, since the blood does not necessarily originate from the lesion itself. If parasites are equally distributed in the sample, as it is the case in peripheral blood of VL patients the PCR seems to be reproducible to a high extent..

4.4. Discussion of PCR results:

4.4.1. Results with primers Uni21/Lmj4:

The PCR using the kDNA primers Uni 21/Lmj4 was found to be useful to distinguish between *L.major* and *L.tropica* infections. The PCR on purified DNA and on cultured organisms proved to be reliable, yielding always a 680 bp product in *L.major* typed strains and a product of 820 bp in *L.tropica* typed strains. Non-leishmanial *Kinetoplastidae* did not amplify. The sensitivity with purified DNA (from cltured promastigotes) was promising, with regard to the potential use for direct diagnosis. Harris *et al.* (1998) estimated that 1 pg of DNA is equal to 10 parasites. The potential sensitivity of primers Uni21/Lmj4 therefore was corresponding to the DNA of 1/4 parasite. Nevertheless, the primer pair Uni21/ Lmj4 was often not sensitive enough to detect single parasites from clinical samples. Efforts have been made to optimize the sensitivity by making the following changes:

1. DMSO and formamid or BSA were added to the PCR in order to block inhibitory activity. Thus more µl of extracted template were tolerated in the reaction.

2. The choice of the polymerase influenced the outcome to a great extent: the Promega Taq polymerase often failed to amplify the 680-850 bp sequence from clinical samples. The TaKaRa Ex Taq and the Fermentas Taq polymerase proved to be more reliable.
3. It was hoped the sensitivity would be improved under less stringent PCR-conditions. A lower annealing temperature (50°C instead of 60°C) yielded also good results. Since occasionally non-specific PCR products of different sizes appeared the original annealing temperature (60°C) was finally adapted as the standard annealing temperature.
4. A higher number of cycles (45 instead of 35) was an alternative option to increase the sensitivity.
5. The staining with Gel Star stain instead of ethidium bromide staining clearly increased the sensitivity. Bands which were nearly invisible with ethidium bromid were distinct and clear when stained with Gel star stain.

The PCR with primers Uni21/Lmj4 showed primarily good results with dermal scrapings on filter paper, and appeared to be a suitable method for direct diagnosis and simultaneous differentiation of *L.major* and *L.tropica*. Even on crude extractions (from dermal scrapings on filter paper), samples were amplified successfully (lysis with NET-buffer and proteinase K digestion). At the beginning, the results were very promising, but later this PCR often failed to produce results, even on microscopically confirmed patients. Nevertheless, whenever the PCR succeeded the results were valuable and very helpful. The results were collected during the developmental stage when conditions of extraction and PCR were often modified. No amplification was probably often due to sub-optimal conditions (too much template eg.). A statistical analysis was therefore not possible. In 6 out of 24 PCR-positive patients the diagnosis was made by PCR only, since smear and culture had been negative.

Specific cases diagnosed with kinetoplast primers Uni21/Lmj4:

1. The case of the patient with the severe nose infection and a possible double infection will be discussed in the following section: controversial results.
2. The diagnosis of *L.major* in the couple from Holon was important to exclude any other causative species. The area is not endemic for leishmaniasis and the source of the infection remained unclear. The couple claimed they had not travelled in- or outside the country since more than one year. The ecology (city, close to the Mediterranean) did not suggest a new focus of *L.major*. The only conclusive hypothesis was that the neighbour had imported infected sandflies in his car. The couple reported the neighbour was going frequently to the Jordan valley by car. The source of the infection remained unclear.

3. The correct identification of *L.major* from a lesion caused by a *L.major* vaccination strain confirmed the specificity of the primers (patient No. 24 in Table No. 6).
4. The results from 4 skin scrapings collected from *Psammomys* ears show that the kDNA PCR with the primer pair Uni21/Lmj4 can be used for detecting infections in reservoir animal species as well.
5. In the case of the infected sandfly from Kfar Adumim *L.tropica* was confirmed with primers Uni21/Lmj4 as soon as promastigotes were discovered in the medium. This case demonstrated the usefulness of primers Uni21/Lmj4 on newly collected strains. Differentiation between *L.major* and *L.tropica* can be achieved long before the normal harvesting of cultures in the stationary phase of growth. Kfar Adumim is a known focus of *Leishmania tropica* (Klaus *et al.*, 1994). It is no contradiction that Kfar Adumim residents were found to be infected with *L.major* as well. Transmission of *L.major* within the settlement Kfar Adumim has not been proven, but it can also not be excluded. The fact that both, *L.major* and *L.tropica* were diagnosed from residents of Kfar Adumim (*L.tropica* also from sandfly derived cultures), emphasizes the necessity of species distinction, even within one settlement. The Jordan Valley (Jericho) as the main transmission area of *L.major* in the country, is very close, the Wadi Kelt being in a distance of less than 1 km. The Bedouins in the area are familiar with CL. The exact limits of *L.major* transmission are not known in the Judean Desert, where Wadis directly connect with the Jordan Valley.
6. A cultured strain obtained from Costa Rica (via Lionel Schnur, Kuvin Center), yielded a PCR-product of 800 bp. Since the *L.braziliensis*- as well as the *L.mexicana*-complex are not amplified with primers Uni21/Lmj4 the only possible New World species amplified with these primers, is *L.d.chagasi*. The fact that the strain was derived from a patient with VL who is living in an area endemic for *L.d.chagasi,* corroborated the diagnosis. The same case has been reported as the first case of VL in Costa Rica by Carrillo *et al,* (1999), *L.d.infantum/chagasi* has been identified by isoenzyme analysis. This demonstrates that the primers Uni21/Lmj4 may be useful in the New World too. *L.d.chagasi* infections could be identified and distinguished from all other New World species. This may be of interest especially for the diagnosis of "atypical CL" in the New World caused by *L.d.chagasi* (Ponce *et al*., 1991).

Controversial results:

In the patient with the severe infection of the nose, the direct PCR diagnosis revealed an infection with *L.tropica* from one lesion, and possibly a double infection (*L.major/L.tropica*) from the other lesion. The PCR on the culture of the same patient some weeks later revealed a

band of 680 bp (*L.major*). The EF performed on the culture identified this isolate as being *L.major*. A contamination of the filter paper extraction is presumably not the cause, since all controls were as expected and all other patient samples in the same series produced the smaller fragment of 680 (*L.major*). The more conclusive explanation is a mixed infection with two species. The origin of the patient (Hemdat) in the bordering area of the northern Jordan Valley and the hilly slopes of Samaria (West Bank) is indeed an area, where both species (*L.major* and *L.tropica*) may occur. Foci of *L.major* (Jordan Valley) and the newly identified focus of *L.tropica* in the hilly area (Wadi Albethan) are both very close. Klaus *et al.* (1994) made similar observations in two patients who had been infected in Kfar Adumim: the PCR (with primers Uni21/Lmj4) had revealed *L.tropica* from the early cultures. The culture had been re-examined after some time, and *L.major* had been identified. It has been observed that the initial growth of the culture was very slow, whereas the later culture was thriving well. The most probable explanation was a mixed infection with two species. It is a known fact that some species thrive better than others in culture. Strejan (1963) observed that different species of *Leishmania* require a different nutritional medium in order to grow well. In a mixed culture one species is therefore gradually outgrowing the other. Harris *et al*., (1998) reported that *L.major* outgrows *L.tropica,* as *L.mexicana* outgrows *L.braziliensis* in mixed cultures. This implies that diagnostic methods based on cultured parasites (isoenzyme electrophoresis, analysis of the EF, other PCR-methods) might be misleading in the case of double infections. As it was the fact in this patient the classical methods would have missed the more problematic causative agent (*L.tropica*), which requires medical intervention. A concurrent infection with *L.major* and *L.donovani* has been reported from Kenya (Mebrahtu *et al*., 1991). It can be assumed that double infections were greatly underreported in the past because diagnosis relied mostly on culture-based techniques. The presented case clearly demonstrates the relevance of direct diagnosis.

It can not be excluded that some strains have characteristics of two species: Oren *et al.*, (1991) reported about a 21 year old Israeli with VL, who came from the area south of the Sea of Galilee. A strain was isolated which was typed as *L.tropica* by EF whereas isoenzyme analysis had revealed characteristics from both, *L.donovani* and *L.tropica.*

The PCR with primers Uni21/Lmj4 can not discriminate between *L.tropica* and *L.d.infantum*. A PCR-product of 800 bp was attributed to *L.tropica*, but theoretically it could be also due to *L.d.infantum*. There is no record of cutaneous *L.d.infantum* strains in Israel and the PA so far, but since it has been reported from other countries (Lebanon, Italy, Tunisia, Iran) it has to be considered (Nuwayri-Salti *et al*., 1994; Gramiccia *et al*., 1989; 1991; Hatam *et al*., 1997, respectively). Endemic areas of *L.tropica* and *L.d.infantum* are partly overlapping in the Jenin district, which makes the differentiation relevant. An attempt to overcome the limitation in

species discrimination with primers Uni21/Lmj4 was performed by Kefaya Azmi and Obeida Yussuf (students of the Al Quds-University, Jerusalem, laboratory work performed at the Kuvin Center), who tested many restriction enzymes for RFLP- analysis of the PCR product and were able to differentiate the species according to their patterns.

4.4.2. Results with the genus specific kDNA-primers 13A/13B:

The genus specific primer pair 13A/13B proved to be highly sensitive, up to 10 fg (1 pg = 10 parasites, Harris *et al*., 1998) and was able to detect probably every single infection. The high sensitivity of the PCR with primers 13A/13B had been also found in previous studies (Rodríguez *et al*., 1994). This PCR proved to be an excellent screening method for the presence of *Leishmania* DNA, directly from dermal scrapings. Noyes *et al*., (1998) discussed, that the amplification of the conserved part (120 bp) of the kDNA minicircles is often sufficient because the species is already known (eg. screening of *Psammomys*). Primers 13A/13B amplified also non-leishmanial *Kinetoplastidae*, such as *Trypanosoma*, which has to be considered if infections are contracted in areas co-endemic with the Chagas disease or sleeping sickness (trypanosomiasis).

The PCR with primers 13A/13B was introduced as a screening method. It has been successfully employed on several sample groups, after the contamination problems were controlled. The specific results will be discussed in extra sections: the results from the Tel Hashomer patients (New World leishmaniasis) will be discussed together with the corresponding results with primers MP3H/MPL1 in the following section, the results from the new *L.tropica* focus Wadi Albethan (West Bank) will be discussed in section 4.5., the animal samples will be discussed in section 4.6.

4.4.3. Results with *L.braziliensis* specific primers MP3H/MPL1:

Since New World leishmaniasis is occasionally seen in Israeli travellers it was important to establish also PCR-diagnosis for the *Leishmania* species of the New World. Because of the risk of mucocutaneous leishmaniasis (MCL, Espundia) later in the case of *L.braziliensis*-infections, the major goal was to identify the infections caused by the *L.braziliensis* complex. The diagnosis of *L.mexicana* species was also desired but of secondary relevance. Primers MP3H/MPL1 were selected because they met the two requirements, being able to distinguish between the *L.braziliensis* and the *L.mexicana* complex and also to detect single parasites (Lopez *et. al*., 1993). The primers MP3H/MPL1 had been previously employed also on dermal scrapings and had been proved to be more sensitive than microscopy (Belli *et. al*., 1998). When tested on reference strains the species of the *L.braziliensis* complex (*L.braziliensis, L.guyanensis,*

L.panamensis) were amplified very efficiently. *L.mexicana* and the species of the Old World (*L.major*, *L.tropica*, the *L.donovani* complex) were not amplified, thus confirming the species-specificity of the primers. The sensitivity of the primers were evaluated empirically on dermal scrapings, in relation to the corresponding results with primers 13A/13B. Anyhow it was important to examine the returners with both primer pairs, since they could have contracted the disease also in Israel, either before or after the journey to America (Table 6, patients 11 and 12!). All 6 patients who had contracted CL in Bolivia were diagnosed with *L.braziliensis* directly from lesion scrapings. One patient had visited Guatemala and was negative with the *L.braziliensis* specific primers but positive with the genus specific primers (13A/13B). This finding suggested an infection by species of the *L.mexicana* complex. *L.d.chagasi* was another possibility since it would have reacted identically with primers MP3H/MPL1 and primers 13A/13B. So far, "atypical" CL caused by *L.d.chagasi* has been reported from Costa Rica, Honduras (Ponce *et al*., 1991) and from Nicaragua (Harris *et al*., 1998; Belli *et al.,* 1999). The other 4 patients who were treated at the Tel Hashomer Hospital had been infected in the Old World. All 4 were negative with the *L.braziliensis* specific primers and positive with the genus-specific primers, as expected. Repeated sampling and PCR, before, directly after and again months after treatment showed that the PCR signal became fainter or even disappeared after healing in most cases. In two patients who were suffering from a clinical relapse (patients 1 and 7, Table 8), the previously faint signals became more intense again. This was interpreted as a confirmation of the relapse, requiring another 3 weeks course of systemic pentostam. Not every PCR-positivity months after completion of the treatment should be regarded as a sign of a relapse. Due to the high sensitivity of the PCR a tiny remnant of DNA may easily be amplified, especially if the targeted sequence is as small as 70 bp. A weak amplification was seen in most of the cases after treatment and progressive healing. The clinical appearance needs to be judged primarily and the PCR-results are usually helpful to confirm the clinical findings. One has to be careful to draw conclusions on the variable quantity of the PCR-product. The reproducibility especially from lesion scrapings is limited, as discussed earlier. The amount of collected tissue was not standardized and was different in every new sample. Nevertheless, a constant decrease of PCR-amplification after completion of the treatment is certainly a good indication for cure. A study conducted by (Nuzum *et al*., 1995) has in fact shown that 12 out of 13 previously PCR-positive VL patients were PCR-negative 6 months after treatment and cure.

Only recently we learned that a follow up study with repeated dermal scrapings and PCR, as it was performed here, may not be advisable due to increased risk of reactivation. This applies especially for *L.braziliensis* infections, which tend to recidivate (personal communication with Palmira Guevara, University of Venezuela, Caracas, Venezuela).

4.4.4. Results with ITS-1 primers and RFLP-analysis:

The ITS-1 amplification combined with RFLP-analysis was published only recently (Schönian *et al*., 2000; El Tai *et al*., 2000), otherwise it would have been adopted as the standard methodology of this thesis from the beginning, due to its species specificity and good sensitivity. With purified DNA it had attained the same sensitivity as with primers Uni21/Lmj4 (1 pg). This PCR method allows species specific diagnosis of all Old World species and all New World complexes. The different species of the *L.braziliensis* complex appear identical, the species of the *L.mexicana* complex (*L.mexicana* and *L.amazonensis*) can be differentiated.

In this study the ITS-1-PCR was applied on already extracted clinical samples which had been stored at -20°C. The restriction of the PCR-product with the enzyme *BsuRI* (*Hae III*) showed the expected patterns, allowing for species specific diagnosis. The results of the pre-diagnosed samples were all confirmed. The ITS-1-amplification in combination with RFLP analysis is clearly superior to the other PCR methods presented here. The additional step of about 2 hours incubation and an additional gel electrophoresis run is a negligible disadvantage in relation to the benefits. Most PCR-systems found in the literature achieve either high sensitivity or species specificity. Only a few are sensitive and also species specific for very few species simultaneously. With this method nearly every *Leishmania* species can be identified with high sensitivity.

4.5. Wadi Albethan, a newly identified focus of *L.tropica* in the West Bank:

A new focus of *L.tropica* has been identified in Wadi Albethan, an Arab village east of Nablus. Probably the whole mountain range west of the Jordan Valley is prone to be endemic with *L.tropica*. The area has not been systematically investigated yet. One soldier reported about several other soldiers being infected with CL in a military camp on the same longitudinal line some kilometers south (personal communication). Kfar Adumim is also on the same longitudinal line as Tiberias in the north (both *L.tropica* foci). The outbreak of CL in Wadi Albethan was confirmed as being caused by *L.tropica* with primers Uni21/Lmj4 from the 2 successfully cultured strains, RFLP analysis of the PCR-product (performed by Kefaya Azmi), ITS-1 amplification and RFLP analysis and the PPIP-PCR (performed by Carol Eisenberger, Kuvin Center, Jerusalem). It proved to be very difficult to retrieve sufficient template DNA from dermal scrapings for the amplification with primers Uni21/Lmj4. The ITS-1-PCR had not been published at that time, which would have been the superior method.

The microscopical examination of the 23 smears was sensitive to 39 %. This confirmed the findings of many other studies, in which the microscopical detection rate did not exceed the

sensitivity of PCR. Four smears of this study group, as well as many others seen during the study period, had to be classified as negative, even though the microscopical picture was highly suggestive of a leishmanial infection. Cell like structures in the size of amastigotes were often seen in groups either intracellularly in macrophages or extracellularly. Nuclei and kinetoplasts could not be identified. In most of these cases patients had been treated previously either with intralesional pentostam or liquid nitrogen. It was assumed that the described structures were destroyed amastigotes. The smears showed a picture of successful destruction of the parasite, which is expected under treatment as well as during a natural healing process.

4.6. Discussion of PCR results from animals:

Desert rodents:

The study on 30 ear scrapings of *Psammomys* was performed in order to approve the reliability of the direct PCR diagnosis. Besides, it was an interesting field study which revealed a high percentage of *Leishmania* infections in the *Psammomys* of the Qziot area in the Negev. The fact that all positive smears (20) were also positive by PCR confirmed the specificity of the PCR (no false negatives). The sensitivity by PCR (28) was far higher than by microscopy (20), as expected. The screening of ear tissue from other desert rodents from the Qziot area revealed a *Leishmania* infection in a rodent species (*Gerbillus dasyarus*), which was not known previously to host the parasite. A new reservoir host species has been identified by PCR. The faint bands of 120 bp indicated the presence of leishmanial DNA. The result was confirmed by one amastigote found in one of the smears. So far, the findings can only prove the infection of this gerbil species; the question if this species is in fact functioning as a reservoir has yet to be elucidated.

Canids:

The peripheral blood from four examined dogs suffering from CVL was PCR-positive with primers 13A/13B. This small study showed that one drop of blood can be sufficient for diagnosis of VL. It can be assumed that VL could be diagnosed equally well also from human blood (discussed later in the section on diagnosis of VL). Thus suitable laboratory tools have been established for the diagnosis of VL too. As mentioned earlier infantile kala azar is occasionally seen in the West Bank and many dogs are infected in Israel (only poor data of dogs in the West Bank) which shows the relevancy.

All jackal ears (of 20 animals) were negative. Most of the western-blot results of the same animals had been either negative or only borderline positive (according to Gad Baneth, Veterinary School, Hebrew University, Rehovot). The examination of the ears had a low chance for positive PCR-results, but it was considered worth-while to try because of the great relevancy

to identify infections in jackals (see Introduction). The jackal spleens in formalin were all negative by PCR. The tissue was not preserved well, which questioned the quality of the extracted DNA anyhow, as mentioned before. Other possible reasons for the negativity could be either due to inhibition of the PCR by remnants of formalin or to the fact that the animals were not infected with *Leishmania*.

Hyraxes as a reservoir of *L.tropica*?:

The examined hyrax tissue (nose, skin and bone tissue of the parched hyrax and the ear biopsy of a living animal) was negative. One hyrax was parched and it was not certain if DNA could be recovered at all. The other hyrax was not exactly from a known endemic area. It was obvious that systematic trapping of hyraxes in a highly endemic area would be necessary in order to detect potential infections. Wadi Albethan would be an excellent study area since it is hyper-endemic for *L.tropica* and hyraxes are abundant. Due to the recent political developments this study can not be carried out presently but the methodology is available now in the laboratory to conduct these studies in the future.

Hyraxes (Photo 19) were observed close to Kfar Adumim (*L.tropica* focus, Photo 13) in the Judean Desert and in Korazim, a settlement on the northern slope of the lake of Galilee, which is one other new *L.tropica* focus in the country (patient 5, Table 8, Photo 3). Also in Jordan, hyraxes were observed in the three major foci of *L.tropica,* and were therefore highly suspected to be the reservoir (Saliba *et al*., 1997). Previous findings have supported the hypothesis: *L.tropica* has been identified in a hyrax in Kenya (Sang *et al*., 1992). Hyraxes were observed in a new focus of *L.tropica* in Kenya (Sang *et al*., 1994). Hyraxes have been found infected with *L.aethiopica* in Ethiopia (Ashford *et al.*, 1973). The cultured parasites had not been characterized at the time of publication. Since *L.tropica* has been isolated from dogs in Morocco (Dereure, 1991) and was also found in a rat (Aljeboori and Evans, 1980), both animal species have to be considered as being a potential reservoir as well. It has to be clarified if dogs or rodents are only accidental hosts or if they are functioning as reservoir.

4.7. Choice of treatment on the basis of PCR-results:

Species- or at least complex-specific identification is essential for the selection of the appropriate treatment. Early differentiation of *L.braziliensis* and *L.mexicana* infections can help to avoid unnecessary hospitalization and treatment of patients infected with *L.mexicana*. If these patients still require treatment, ambulant care would be sufficient and drugs (ketokonazol eg.) could be administered orally (Rodriguez *et al*., 1994). Several patients in Israel might be spared from toxic and costly treatment every year. A three weeks course of intravenous pentostam in Israel

sums up to about $ 10,000 for hospitalization and 500 $ for the treatment (20 mg/kg/day pentostam for 20 days) (personal communication with Dr. Eli Schwartz, Tel Hashomer Hospital, Tel Aviv).

Infections with *L.mexicana* and the *L.braziliensis* complex were found to respond differently to certain drugs (Navin *et al*., 1992). 90 % of patients with *L.braziliensis*-infections responded well to sodium stibogluconate whereas only 57% of *L.mexicana* infections showed improvement. Using ketoconazol 89% of patients with *L.mexicana* infections responded well compared to only 30% of the patients infected with *L.braziliensis*. The PCR results with primers Uni21/Lmj4 can also give a direction for the choice of the adequate therapy. If *L.major* is diagnosed (680 bp), usually no treatment is required. If a >800 bp product is found the patient needs to be treated (*L.tropica, L.donovani* complex). The ITS-1 amplification combined with restriction is evidently a suitable method for species specific diagnosis and therefore provides the information necessary to treat a patient adequately.

4.8. PCR diagnosis in VL:

This study focused on CL because VL is rare and most cases occur in the West Bank (Photo 14), where the access to doctors and hospitals is complicated. Even if there were single cases in the past two years they were not necessarily referred to our laboratory. The methodology which has been successful for the diagnosis of CL can be applied as well for the diagnosis of VL. As the small study on 4 infected dogs has shown, VL can be diagnosed directly from peripheral blood. It is of great importance that these methods are available in the country, especially for the diagnosis of human (infantile kala azar) in the West Bank but also for epidemiological studies in canids. The methods are suitable for a clinical laboratory, which is equipped with a PCR machine. Osman *et al*., (1997 a) evaluated PCR-diagnosis on peripheral blood spots from confirmed and suspected VL patients in Sudan. The PCR was positive in 70% of the confirmed and in 19% of the suspected cases. In the same study, also different clinical specimens were compared for their detection rate by PCR. Lymph node aspirates, bone marrow aspirates and peripheral blood (finger-prick-blood) of microscopically confirmed VL cases in Sudan were collected on filter paper and extracted with phenol-chloroform. The PCR revealed positivity in 100% of the bone marrow aspirates, in 87% of the lymph node aspirates and in 70% of the peripheral blood samples. Even though the detection rate from blood was the least, as expected, it is an excellent method for first line screening in suspected cases of VL. Thus many patients could be spared from the more invasive diagnostic methods, such as bone marrow- and splenic aspirations. Also Campino *et al*., (2000) have found a good detection rate with peripheral blood

spots on filter paper. Out of 20 immunocompromised and previously diagnosed VL patients 15 were confirmed by PCR (SSU-rRNA primers).

The use of the buffy coat (Lachaud *et al*., 2001) or separated peripheral blood mononuclear cells (PBMC) (Nuzum *et al*., 1995 and Belli *et al*., 1998) may be even more promising since the parasites are concentrated in these cells. The cells (PBMC) were separated from peripheral blood and only crudely prepared (lysis in a buffer, digestion with proteinase K and boiled for 15 minutes). Belli *et al*., (1999) found 7 out of 9 VL patients to be positive by PCR. Adya *et al*., (1995) compared the sensitivity of PCR, ELISA and microscopy in Indian patients with symptoms suggestive of VL. Out of 22 patients 17 were positive by PCR, 14 by ELISA and 7 by smears which had been prepared from spleen aspirates.

Since the reservoir and the vector for VL are prevalent in many places in Israel and in the West Bank, CVL is increasingly seen in domestic dogs in Israel, outbreaks in humans could occur as well at any time. It is therefore good to be technically prepared for reliable diagnosis. Serology is available, but antibodies are not necessarily found in the beginning of the disease. Diagnosis from spleen or bone marrow aspirates is also very sensitive, but can be left for the PCR-negative cases (from peripheral blood).

4.9. Guidelines for future applications:

4.9.1 Sampling, preservation and extraction:

As a result of the observations made during the whole study period and the findings of the comparative study (with Carney Mattheson, not included in this thesis), the following proceeding can be recommended for a clinical laboratory: Dermal scrapings on filter paper are very suitable samples which can be highly recommended for the PCR-diagnosis of CL. For the diagnosis of VL, the buffy coat of peripheral blood appears to be the best specimen, which can be spotted onto filter paper as well. Bone marrow aspirations (more sensitive but also more invasive) can be preserved as second line diagnostic measures.

Filter paper samples consisting mostly of tissue and only of a small amount of blood can be extracted with the Chelex method with excellent results. If specimens contain a larger amount of blood (eg. also dermal scrapings on filter paper, which accidently consist mainly of a drop of peripheral blood) or are conserved differently (fixed in formalin, paraffin embedded, Giemsa stained) the phenol-chloroform extraction should be used. The guanidine method can not offer more than the Chelex method and may be preserved for exceptional samples (bone powder, macerated tissue). It may be considered for samples in which a certain degree of purification is necessary and a loss of material (as it can not be avoided with the phenol-chloroform extraction) would be critical.

The problem of inhibition has to be considered. Inhibition is influenced by the original sample size in relation to the volume of lysis, extraction and DNA resuspension and also by the amount of template DNA used in the PCR. It is self-evident that the way of preservation plays an important role as well as the removal of inhibitory substances during DNA extraction, which depends mainly on the employed extraction method. In the case of dermal scrapings on filter paper in the size of about 0.5 cm in diameter all three methods are efficient (phenol-chloroform, guanidine and chelex).

4.9.2. Recommended approach for PCR diagnosis in the future:

The amplification of the ITS-1 region should be the method of first choice. No other system has been found in the literature so far which is simultaneously as sensitive and species specific for the whole spectrum of *Leishmania* species. It is of great benefit especially for a country like Israel, where 3 species are endemic and any species may be imported. Therefore any *Leishmania* species can potentially be encountered in Israel. In countries where only one species is known to be endemic it would be sufficient to amplify the ITS-1 region for a genus specific result without consecutive RFLP-analysis. RFLP-analysis could follow if the disease was imported from another country. If a laboratory prefers to concentrate only on genus specific diagnosis, kinetoplast primers 13A/13B should be favoured due to the higher sensitivity. These primers may be employed for the same reason in cases of negativity with the ITS-1 primers. With the advent of the ITS-1 primers the *L.braziliensis* specific primers MPL1/MP3H can be replaced as well. If the ITS-1 amplification is negative, they may be used in a second attempt (higher sensitivity). The ITS-1 primers are superior in New World leishmaniasis because they can identify also *L.mexicana* infections, whereas the *L.braziliensi*s primers can positively identify only species of the *L.braziliensis* complex.

Kinetoplast primers Uni21/Lmj4 can be replaced by ITS-1 primers due to the higher sensitivity of the latter. There may be still applications where primers Uni21/Lmj4 are benefitial: Whenever a distinction between *L.major* and *L.tropica* is required and enzyme digestion (with the ITS-1 method) is not wanted or not possible the kinetoplast primers can be employed. It can be assumed that primers Uni21/Lmj4 could have a high success rate if the best conditions (discussed earlier) are chosen on a regular basis.

4.10. Leishmaniasis in non-endemic areas with particular regard to Germany:

In Germany leishmaniasis is occasionally seen in travellers and immigrants. The incidence of leishmaniasis is not known since the disease is not reportable. Due to tourism the number of

cases is increasing. According to the "Deutsches Ärzteblatt" (Harms-Zwingenberger and Bienzle, 2000) patients are either treated by local physicians or in hospitals. Diagnosis relies on microscopy, culturing, serology and PCR. Rarely cases of infantile kala azar have been diagnosed in the past. One interesting case was reported recently: an infant of 15 months was diagnosed with VL, who had never left Germany except for a short visit to Holland (Bogdan *et al*, 2001). After 6 months of fever and hepatosplenomegaly kala azar was finally diagnosed. The parents reported the family had spent holidays at a campsite in southern Germany (Füssen) in the previous summer, where many returners from the Mediterranean use to camp on their way back to Germany. One hypothesis suggested that an infected sandfly might have been imported in a car. It was also suggested that autochthonous foci could possibly emerge in central Europe. This is indeed worth-while to consider as a realistic possibility since sandflies have been identified recently in south-west Germany along the upper Rhine valley. The identified sandfly species, *Phlebotomus mascittii* Grassi 1908, belongs to a subgenus responsible for transmission of VL in the Mediterranean (Naucke and Pesson, 2000). The prospect of a warmer climate in Europe may create conditions for endemic leishmaniasis in central Europe (Kuhn, 1999).

Dogs are much more frequently diagnosed with CVL in Germany than humans with VL. According to Gothe *et al*., (1997) 236 dogs were diagnosed with leishmaniasis between 1993 and 1995. The dogs were either imported from Mediterranean countries or had been travelling with their owners. It can not be excluded that infected dogs could potentially introduce *L.d.infantum* and function also as a reservoir in an emerging focus.

Individual cases have shown, that it is worth-while to search for leishmaniasis in patients with fever of unknown origin (FUO) and splenomegaly, even if the patient history does not suggest kala azar. Unusual ways of transmission have been reported as for example congenital transmission (Meinecke *et al*., 1999), blood transfusion (Le Fichoux *et al*., 1999) and possibly also liver transplantation (Grimm *et al*., 1996). Furthermore, one has to be aware that immunosuppressed persons (eg. HIV-infected persons, transplant recipients, patients with chronic diseases) are at risk to contract VL during visits to Mediteranean countries. It should be also seriously considered to screen transplant organs routinely by PCR if they come from endemic countries in Europe. As transplantation is increasingly organized centrally in Europe this measure could help to prevent cases of “autochthonous VL” in non-endemic European countries. Another interesting fact should be notified: it has been shown by Le Fichoux *et al*., (1999) and it has been dicussed also by Kubar *et al*., (1997) that the rate of subclinical infections, even with parasitemia, can be considerably high in endemic areas of kala azar (eg. southern France, Nice area). This is a relevant issue with regard to blood transfusion in these areas. Out of 565 blood donors who were screened for the presence of antibodies (by western blot) 76 were

found to be seropositive. The buffy coats of the positive samples were cultured and promastigotes were detected in 9 cultures. Grogl *et al*, (1993) have studied the survivability of *Leishmania* parasites in human blood products under blood bank conditions. It was found that the parasites survive the conditions up to at least 25 days and also retain their infectivity. Also tourists to the Mediterranean could contract subclinical infections, and could become symptomatic after years, eg. in a state of immune suppression. The same applies for residents who come from Mediterranean or Middle Eastern countries (eg. Turks, Iranians, Arabs) In non-endemic countries physicians are often not aware of leishmaniases and diagnosis is usually delayed, especially in the serious cases of kala azar with unusual histories. Since infections can theoretically be caused by all *Leishmania* species the identification at the species level is mandatory. Sensitive direct PCR diagnosis has the same relevancy as in endemic regions and should be favoured as the first diagnostic measure in suspected cases of VL and should be routinely performed in cases of CL in addition to microscopy. Culture based methods and serology can be performed as well but should be second line diagnostic measures. Invasive diagnostic measures, such as skin biopsies (CL) or bone marrow aspirations (VL), can be preserved for the case that the primary PCR on dermal scrapings (CL) or peripheral blood/ buffy coat (VL) is negative and symptoms still support the diagnosis.

4.11. Concluding remarks:

As leishmaniasis is not defeated but is rather a health threat more than ever before, improved diagnosis is of crucial importance for any attempt to control the disease. Diagnostic methods have to be simple and cheap enough to be used also in developing countries, and they have to be suitable for field work too. Endemic areas are not confined to the developing world, as most of the classical tropical diseases are. Also the developed world has to be prepared to see more patients with leishmaniasis, mostly due to endemicity in many countries with a warmer climate, travelling, but also as an opportunistic disease in HIV-infected patients. PCR is a very powerful tool for sensitive and specific diagnosis. The benefits of the PCR-methodology are undoubtedly enormous. This work has focused on PCR-diagnosis of leishmaniasis in Israel and the West Bank, where the methods have been established and are ready now for routine use. This study has been also exemplary for other endemic or non-endemic countries, where direct PCR diagnosis of leishmaniasis is planned to be introduced. The methods can be adopted, benefits of different extraction- and PCR-methods can be estimated with the help of this research and the best combination of methods can be selected for different settings in other countries.

5. Summary

In Israel and the West Bank, three species of *Leishmania* are prevalent: *L.major, L.tropica and L.donovani infantum* (*L.d.infantum*). Cutaneous leishmaniasis (CL) is mostly caused by *L.major* in the Negev and the Jordan Valley. Its ecoepidemiology has been studied thoroughly in the past. CL due to *L.tropica* is more sporadic, but new foci have been emerging in recent years in the northern, more hilly parts of the country. The epidemiology is only poorly understood because only very few of the *L.tropica* foci within Israeli territory have been investigated so far. Only few data are available on *L.tropica* foci within the West Bank. The transmission is highly suspected to be zoonotic, but the reservoir animal species has not been identified yet. Since *L. tropica* infections may visceralize or become chronic (lupoid leishmaniasis) they need to be distinguished from *L.major* infections. Any emerging focus of CL therefore requires species specific identification.

L.d.infantum causes visceral leishmaniasis (VL) in humans and in canids in Israel and the West Bank. Human disease is mostly seen in infants in the West Bank (Jenin district). It is not frequently diagnosed but it poses a serious life threat, and outbreaks are theoretically possible at any time. In Israel, the disease predominantly affects dogs. Canine visceral leishmaniasis (CVL) is diagnosed more frequently than ever before in the country. It is an emerging disease, which is spreading in central Israel and is also a potential danger for humans. In the northern West Bank, *L.tropica* and *L.d.infantum* foci are overlapping. Since strains of *L.tropica* were reported to visceralize and *L.d.infantum* strains may be dermatotropic, it is obvious that the differentiation of the two species would be of clinical and epidemiological importance.

Apart from the endemic forms of leishmaniasis, imported forms, especially from the New World, are occasionally seen in Israel. Many young Israelis travel to Central and South America, and a few of them contract CL every year. Species-specific diagnosis is of great importance especially in these patients since only *L.braziliensis* infections require hospitalization and intravenous therapy with antimonal drugs.

The aim of this work was to establish field applicable methods for the diagnosis of leishmaniasis directly from clinical material. Furthermore, it was important to introduce species-specific diagnosis for the above-named reasons. Apart from improved diagnosis of patients, screening methods for epidemiological studies were needed. Dermal scrapings were collected from cutaneous lesions of patients and of rodents. The samples were preserved on sterile filter paper. Several DNA extraction methods were compared, ranging from thorough to crude purification. Apart from dermal scrapings, various other clinical specimens were successfully proceeded

(Giemsa stained smears and paraffin embedded biopsies). This showed that Giemsa stained smears and paraffin embedded biopsies are suitable for retrospective studies, if required. For skin scrapings from desert rodents, the chelex-extraction yielded excellent results. For the screening of many samples, the work can be facilitated to a great extent by using chelex-extraction instead of the phenol-chloroform or the guanidine extraction method. It was found that samples with a relatively high tissue and a low blood content yielded excellent results with the chelex method. If samples were bloody, the phenol-chloroform extraction was superior. Furthermore it was shown that inhibition of the PCR by interfering factors (predominantly hemoglobin) could be circumvented by using additives in the PCR, such as bovine serum albumin (BSA), formamide and dimethoxysulfoxid (DMSO). The DNA was subjected to different polymerase chain reactions (PCR), which targeted the kinetoplast minicircle DNA (kDNA) and later also another region, the intergenic transcribed spacer (ITS-1) within the ribosomal operon. The study primarily focused on primers Uni21/Lmj4, which amplified whole kDNA minicircles of the Old World species of *Leishmania*. These primers had never been tested systematically and the sensitivity was not known. *L.tropica/L.infantum* and *L.major* infections were distinguished by species-specific sizes of the minicircles. This PCR method was useful for the direct diagnosis of cutaneous leishmaniasis in individuals, but was not reliable enough to be employed for routine diagnosis. The genus-specific kDNA primer pair 13A/13B, which amplifies a conserved sequence of 120 bp, was highly sensitive and was successfully employed as a screening method in humans and in reservoir animals. Primers MP3H/MPL1 amplified a 70 bp sequence specific for the *L.braziliensis* complex. *L.braziliensis* infections were diagnosed in 7 patients using these primers. The course of the disease was monitored by PCR over several months. Only recently has species-specific diagnosis by restriction fragment length polymorphism (RFLP) analysis of the amplified ITS-1 region (~300 bp) been published (El Tai *et al*., 2000) and was adopted in our laboratory. It provided sensitive and species-specific diagnosis of both the Old World and New World species, and therefore covered all the requirements simultaneously.

During the study, a new focus of *L.tropica* was identified in the West Bank, in Wadi Albethan, a village east of Nablus. A survey of desert rodents trapped in the Negev revealed a new rodent species (*Gerbillus dasyarus*) as a possible host for *Leishmania* parasites. The diagnosis of *L.braziliensis* infections was achieved for the first time in the country. Species-specific diagnosis directly from clinical samples is now available in Israel. The methods are ready to be introduced in other laboratories in the country or elsewhere, and have been adapted for possible routine use in clinical laboratories. They are suitable for diagnosis as well as for epidemiological studies.

Zusammenfassung

Die Leischmaniose ist endemisch in Israel und in den palästinensischen Gebieten (West Bank). Drei Arten von Leishmanien kommen in Israel vor, *L.major*, *L.tropica* und *L.donovani infantum* (*L.d.infantum*), welche im wesentlichen zwei Krankheitsbilder verursachen: Die kutane Leischmaniose, oder auch Orientbeule, wird überwiegend von *L.major* verursacht, und eher seltener, jedoch in zunehmendem Maße auch von *L.tropica*. Die viszerale Leischmaniose, oder auch Kala Azar, wird in Israel von *L.d.infantum* hervorgerufen.

Die Epidemiologie von *L.major*, der häufigsten und auch harmlosesten Spezies im Land, ist weitgehend erforscht. *L.major* ist vor allem in den Wüstengegenden des Landes verbreitet (Negev, Arava), im besonderen auch in Jericho („Jerichobeule“) und im Jordantal. *L.major* kommt als eine Zoonose vor, Wüstennagetiere wie z. B. die fette Sandratte (*Psammomys obesus*) dienen als Reservoir. *L.tropica* ist vergleichsweise wenig erforscht. Diese Spezies kommt eher sporadisch im nördlichen, höher gelegenen Teil des Landes vor (Galiläa, Samaria, Westjordanland). Immer wieder sind in den letzten Jahren neue Ausbrüche aufgetreten, von denen die meisten noch wenig erforscht, geschweige denn mit Sicherheit identifiziert worden sind (vor allem im Westjordanland). Es wird angenommen, daß es sich auch bei den *L.tropica* Infektionen um eine Zoonose handelt (im Unterschied zur bekannten Anthroponose bei *L.tropica* in den größeren Städten des Nahen Ostens), jedoch ist das tierische Reservoir bislang nicht bekannt. Da *L.tropica* Infektionen insgesamt schwerwiegender sind, in seltenen Fällen auch zu einer viszeralen Manifestation, oder auch in eine chronische, lupoide Form führen können, müssen sie von *L.major* Infektionen differenziert werden. Insbesondere ist es wichtig, daß neu auftretende Foci spezies-spezifisch identifiziert werden.

Die viszerale Leischmaniose ist ebenfalls ein ernstzunehmendes Problem im Land: Die Erkrankung ist tödlich, wenn sie nicht rechtzeitig diagnostiziert und therapiert wird. Vor allem sind Kleinkinder im nördlichen Westjordanland betroffen. Die Inzidenz ist nicht hoch, jedoch gibt es offensichtlich einen funktionierenden Infektionszyklus, der die Voraussetzung ist für mögliche Ausbrüche der Erkrankung in der Zukunft. In Israel sind bislang nur sehr wenige Fälle von Kala Azar aufgetreten, doch ist in den letzten Jahren deutlich geworden, daß die viszerale Leischmaniose bei Hunden weit verbreitet ist und daher auch für den Menschen eine potenzielle Gefahr besteht. Da das klinische Bild nicht eindeutig auf den Errerger hinweist (*L.d.infantum* kann sich in seltenen Fällen kutan, und *L.tropica* kann sich viszeral manifestieren), ist auch hier eine spezies-spezifische Diagnostik geboten.

Neben den endemischen Formen der Leishmaniose kommen mit zunehmender Häufigkeit auch eingeschleppte Leishmaniosen aus Zentral- und Südamerika vor. Es ist üblich, daß junge Israelis

nach ihrer Entlassung aus der Armee für einige Monate reisen (als Rucksacktouristen). Mit besonderer Häufung werden Infektionen aus dem bolivianischen Regenwald (*L.braziliensis*) beobachtet. Da die Infektionen durch *L.braziliensis* mit einem besonderen Risiko verbunden sind (mögliche spätere mukokutane Leischmaniose, Espundia), müssen diese Patienten für drei Wochen systemisch mit Pentostam behandelt werden (wie bei Kala Azar). Dies ist selbstverständlich mit hohen Kosten verbunden (Hospitalisierung), außerdem ist die Therapie nebenwirkungsreich. Bislang wurden alle Reiserückkehrer aus Amerika, bei denen eine kutane Leischmaniose aufgetreten war, ohne eine spezies-spezifische Diagnostik vorsichtshalber therapiert.

Ziel der vorliegenden Arbeit war es, eine sensitive und spezies-spezifische PCR Diagnostik der Leishmaniose im Land zu etablieren. Neben einer verbesserten Diagnostik bei Patienten war es wichtig, auch Methoden für epidemiologische Studien (z.B. screening von Reservoirtieren) zur Verfügung zu stellen. Für die PCR-Diagnostik wurden vor allem Wundabstriche aus Läsionen (von Patienten und auch Tieren) mithilfe einer sterilen, chirurgischen Klinge gewonnen und auf Filterpapier konserviert. Daneben wurden auch andere Proben bearbeitet und für ihre Eignung zur PCR-Diagnostik geprüft, z. B. Giemsa gefärbte Abstriche, Paraffin eingebettete Biopsien (für eventuelle retrospektive Studien in der Zukunft). Auch wurde peripheres Blut von erkrankten Hunden untersucht (1 Tropfen auf Filterpapier), um gleichzeitig auch die Diagnostik der viszeralen Leischmaniose voranzutreiben.
Um die Diagnostik zu vereinfachen (Anpassung für ein nicht forschungsorientiertes Labor), wurden verschiedene Extraktionsmethoden ausprobiert und ihre Eigenschaften näher untersucht. Alle getesteten Methoden (Phenol-Chloroform, Guanidin, Chelex, „Crude“) waren erfolgreich, d.h. PCR-Resultate wurden direkt von klinischem Material gewonnen. Interessanterweise wurden gerade mit einer sehr einfachen und schnellen Methode (Chelex) exzellente Resultate erzielt (28 von 30 *Psammomys*-Ohr-Proben waren positiv). In diesem Falle waren jedoch auch die Proben ideal (überwiegend Gewebe und wenig blutig). In anderen Fällen zeigte sich, daß PCR-Inhibition ein wesentliches Problem sein kann und daher differenziert werden muß, welche Proben einer gründlichen Aufreinigung bedürfen (Phenol-Chloroform-Extraktion). Es wurden Versuche unternommen, die PCR-Hemmung zu verhindern, indem zusätzliche Substanzen der Reaktion beigefügt wurden (Bovines Serumalbumin, Dimethylsulfoxid, Formamid). Dadurch konnten störende Substanzen, wie z.B. Hämoglobin, tatsächlich effektiv abgefangen werden, sodaß dann die PCR-Reaktionen auch bei grob zubereiteten Proben (z.B. nur Proteinase-K-Verdau plus Lyse) durchaus möglich war.

Für die PCR wurden verschiedene Oligonukleotid-Paare eingesetzt. Zunächst wurde das Problem der Differenzierung von *L.major* und *L.tropica* angegangen, da dies eine vorrangige Aufgabe war. Zu diesem Zweck wurden die eigens dafür entwickelten Primer Uni21/Lmj4 (Eresh *et al*., 1993) getestet. Diese Primer amplifizieren ganze „Minicircle" der Kinetoplasten-DNA (kDNA), die in 10.000 Kopien pro Organismus vorkommen, und daher potenziell als sehr sensitiv eingestuft wurden. Diese Primer-Paar konnte tatsächlich *Leischmania*-DNA direkt aus klinischem Material amplifizieren, die Sensitivität war jedoch insgesamt nicht zuverlässig genug für eine mögliche Einführung als Routinemethode. Für epidemiologische Anwendungen wurde das genus-spezifische Primer Paar (13A/13B, ebenfalls kDNA-Primer, Rodgers *et al*., 1990) verwendet, welches sich als hoch sensitiv erwies, jedoch keine spezies-spezifische Diagnostik leisten konnte.

Die Diagnostik der importierten Leischmaniosen aus der Neuen Welt erforderte den Einsatz eines weiteren Primer-Paares. Um eine hohe Sensitivität zu gewährleisten wurde dafür ebenfalls ein kDNA-Primerpaar eingesetzt (MP3H/MPL1, Lopez *et al*., 1993), welches die Spezies des *L.braziliensis*-Komplexes spezifisch amplifizieren, daneben jedoch keine andere Spezies erkennt. Dadurch konnten mehrere *L.braziliensis*-Infektionen bei Reiserückkehrern identifiziert werden. Um gleichzeitig auch die Infektionen zu erfassen, die nicht durch *L.braziliensis* verursacht waren, wurden dieselben Patientenproben auch mit den genus-spezifischen Primern untersucht. Gegen Ende der experimentellen Arbeit wurde eine vierte Methode eingeführt (El Tai *et al*., 2000), die eine sowohl sensitive als auch gleichzeitig spezies-spezifische Diagnostik für fast alle Spezies ermöglichte. Diese Primer (LITSRn/L5.8S) amplifizieren eine 300 bp Sequenz des ribosomalen Operons (Intergenic transcribed spacer, ITS-1). Diese Sequenz besteht aus konservierten und variablen Anteilen. Die konservierten Anteile ermöglichen eine Amplifikation aller Spezies (genus-spezifisch). Die variablen Anteile der Sequenz (spezies-spezifisch) werden für die spezies-spezifische Diagnostik genutzt, mithilfe von Restriktions-Fragment-Längen-Polymorphismen (RFLP-Analyse). Zwar ist der Restriktionsverdau des PCR-Produktes ein zusätzlicher Schritt, doch wird dieser durch die Überlegenheit der Methode völlig gerechtfertigt.

Während der experimentellen Phase sind „nebenbei" recht interessante Ergebnisse erzielt worden: Es wurde ein neuer *L.tropica*–Fokus identififiziert. In einem palästinensischen Dorf in der Nähe von Nablus (Wadi Albethan, Westjordanland) wurde ein Neuausbruch der kutaner Leischmaniose beobachtet, von dem ein großer Teil der Bevölkerung betroffen war (keine genauen epidemiologischen Daten). Die PCR von Wundabstrichen auf Filterpapier bewährte sich in dieser ersten realen Feldstudie.

In einer umfangreicheren Studie an Wüsten-Nagetieren (Ohr-Wundabstriche auf Filterpapier) wurde eine neue Tierspezies als mögliches Reservoir gefunden, die vorher nicht als solche bekannt war (*Gerbillus dasyarus*). Die genus-spezifische PCR war schwach positiv bei mehreren Individuen, 1 amastigoter Parasit wurde in einem Giemsa gefärbten Ausstrich nachwiesen (Bestätigung der PCR-Ergebnisse).

Direkte (d.h. von klinischem Material, nicht von Kulturen), spezies-spezifische PCR-Diagnostik der Leischmaniasis ist in Israel zum ersten Mal durchgeführt und jetzt etabliert worden, *L.braziliensis* Infektionen sind zum ersten Mal spezies-spezifisch im Land nachgewiesen worden. Die Erfahrungen aus dieser Forschung können helfen, geeignete Konservierungs-, Extraktions- und PCR-Methoden für die Diagnose der Leischmaniose auch in anderen Labors zu übernehmen, sowohl in endemischen als auch in nicht-endemischen Ländern.

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8. CV

THE KUVIN CENTER
FOR THE STUDY OF INFECTIOUS
AND TROPICAL DISEASES
The Hebrew University
Hadassah Medical School
P.O.B. 12272
Jerusalem 91120
Israel
Email: girlande@web.de

BIRTH NAME:		GERLIND ANDERS
DATE OF BIRTH:		18.02.1968
PLACE OF BIRTH:		WÜRZBURG/ GERMANY
PRESENT:	from 1.10.1998	Working at the Kuvin Center for the Study of Infectious and Tropical Diseases, Hadassah Medical School, Hebrew University
GRADUATION:	17.04.1998	3rd. State exam
INTERNSHIP:	4-8/1997	Surgery at the Hadassah Hospital, Hebrew University Ein Karem, Jerusalem, Israel
	8-11/1997	Internal Medicine at the Auguste-Viktoria-Hospital in Berlin
	12/1997-3/1998	Pediatrics at the Rudolf-Virchow Hospital, Charité, Humboldt University, Berlin
	4/1997	2nd. State exam
	9/94-6/96	Working part time at the Max-Delbrück-Center as a medical technician in Molecular Biology
	10/1994	Continuation of medical studies at the Free University of Berlin
	1992-1994	Training as a medical technician at the Philipps University of Marburg
UNIVERSITY:	1989-1992	Medical studies at the Free University of Berlin
SCHOOL:	1974 - 1987	Freie Waldorfschule, Marburg

9. List of publications:

Gerlind Anders, Carol L. Eisenberger, Flory Jonas, and Charles L. Greenblatt Distinguishing *Leishmania tropica* and *Leishmania major* in the Middle East by polymerase chain reaction using kinetoplast DNA specific primers. *Trans. Trop. Med. Hyg.,* suppl., in press.

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Wasserberg G, **Anders G,** Abramsky Z, Kotler B.P., El Fari M, Schoenian G., Schnur L, Warburg A. The eco-epidemiology of cutaneous leishmaniasis in thewestern Negev, Israel: spatial heterogeneity in host prevalence and its underlying ecological mechanisms. *Internat. J. Parasitol.,* submitted 2001

Erklärung an Eides Statt

Hiermit erkläre ich, daβ ich die Dissertation selbst und ohne unzulässige Hilfe Dritter verfaβt habe. Die Arbeit enthält, selbst in Teilen, keine Kopien anderer Arbeiten. Die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.

Berlin, 18.09.01

Gerlind Anders